

The Impact of miRNA-155 Expression on Treatment Outcome in Adult Acute Myeloid Leukemia Patients

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Abstract

Background: Acute myeloid leukemia is a heterogeneous hematologic malignancy associated with gene mutations, chromosomal rearrangements, deregulation of gene expression and epigenetic modifications. The treatment outcome of AML is highly variable signifying the heterogeneous nature of the disease. **Aim of the Study:** To evaluate miRNA-155 expression level as a prognostic marker for adult patients with acute myeloid leukemia. **Patients and Methods:** 101 subjects were included in this study. They were classified into 2 groups, patient group (61 adult patients with newly diagnosed acute myeloid leukemia) and control group (40 apparently healthy adult subjects). miRNA-155 expression was assessed using real time PCR using QIAGEN, miScript, Quanti Tect and Rotor-isc (QIAGEN Group) PAXgene (pre Analytix GmbH). Samples were either peripheral blood or bone marrow aspiration sample. **Results:** Roc curve detected 2.85 as best fit value of miRNA-155 for discriminating patients from healthy controls with sensitivity 92.3%, specificity 88.5%, AUC 0.98 and CI (0.96 - 0.99) ($p < 0.001$). The 75th percentile value of the patient group was taken as prognostic cut off value with a value < 9.8 as low miRNA-155 and a value ≥ 9.8 as high miRNA-155. The expression level of miRNA-155 was significantly higher in AML patients than in controls ($p = 0.002$). Patients with high miRNA expression had a significantly higher white blood cells count ($p = 0.002$), bone marrow blasts ($p = 0.006$) and peripheral blood blasts ($p = 0.006$) compared to patients with low miRNA-155 expression. Patients with poor cytogenetics had a significantly higher level of miRNA-155 expression compared to patients with favorable cytogenetics ($p = 0.007$). The complete remission rate was significantly higher in patients with low miRNA-155 expression compared to those with high expression (86.4%

and 23.5%, consequently, $p = 0.004$). The disease free survival was significantly shorter in patients with high miRNA-155 expression compared to those with low expression (median 12 months, 95% CI (7.4 - 15.5) and median notreached, consquently, $p = 0.001$). The overall survival was significantly higher ($p = 0.002$) in patients with low miRNA-155 expression (median overall survival was not reached) compared to patients with high miRNA-155 expression (median overall survival 14.5 months, 95% CI; 10.2 - 17.6). **Conclusion:** The expression level of miR-155 was significantly higher in AML patients than in control groups and high miRNA-155 expression level was significantly associated with poor cytogenetics, poor response to therapy and shorter disease free and overall survival conferring a poor outcome.

Keywords

miR-155, Micro-RNA, AML

1. Introduction

MicroRNAs (miRNAs) are a class of evolutionarily conserved and single-stranded non-coding RNA molecules of 19 - 24 nucleotides that control gene expression at a post-transcriptional level. They derive from the metabolic processing of long RNA transcripts encoded by miRNA genes. They have been detected in plants, animal species, and viruses and are involved in numerous cellular processes including proliferation, differentiation, apoptosis and metabolism [1].

Studies have shown the potential role of miRNA-155 in oncogenesis of lymphomas, chronic lymphatic leukemia and some solid tumors [2] and the association with pre-B acute lymphatic leukemia and myeloproliferative neoplasms [3] [4].

The expression of miRNA-155 is low in normal hematopoietic cells and often upregulated in AML, where it likely mediates aberrant cell differentiation and proliferation [5].

MiRNA-155 expression is an independent prognostic marker for patients with cytogenetically normal acute myeloid leukemia (AML). Patients with higher miRNA-155 expression experience significantly lower rates of complete remission and shorter disease-free and overall survival [6].

These findings highlight the potential role of miRNA-155 expression level not only as a prognostic factor in AML, but also as a therapeutic target as some compounds with anti-microRNAs are being developed for clinical use [7].

Van Roosbroeck *et al.* [8] demonstrated that overexpression of miR-155 induced resistance to chemotherapy, which could be reversed upon miR-155 inhibition. However, limitations to this treatment include the instability of free-floating anti-miRNAs in the plasma and their vulnerability to breakdown by nucleases, tissue uptake and renal clearance [9]. These limitations may be overcome by nanoparticle-based delivery of the anti-miRNAs to target tissues [10]. In a study

by Babar *et al.* [11], systemic delivery of antisense peptide nucleic acids encapsulated in unique polymer nanoparticles inhibits miR-155 and slows the growth of these “addicted” pre-B-cell tumors *in vivo*, suggesting a promising therapeutic option for lymphoma and leukemia.

2. Patients & Methods

This study was carried out in the Clinical Pathology and Medical Oncology & Hematology Departments, Faculty of Medicine, Zagazig University Hospitals from 5/2015 to 2/2018 on 101 subjects classified into two groups: control group (40 normal individuals) and patient group (61 adults with newly diagnosed AML patients). All patients were diagnosed using standard methods including CBC, Liver & Kidney functions, LDH level, B.M aspiration (morphological examination by Leishman stain & cytochemistry), Immunophenotyping and Cytogenetics.

◆ Patient Inclusion Criteria:

- Age ≥ 18 years, newly diagnosed AML patients.
- No chronic illness or organ failure that would otherwise preclude receiving standard induction protocol.

◆ Patient Exclusion Criteria:

- Patients diagnosed with promyelocytic leukemia.
- Patients who were diagnosed with other malignancies at any time period.
- Previous exposure to chemotherapy or radiotherapy.

All patients received the standard cytarabine-doxorubicin-based induction chemotherapy regimen 3 & 7 consisting of cytarabine 100 mg/m²/day continuous infusion for 7 consecutive days plus doxorubicin 45 mg/m²/day for 3 days.

Written informed consent was taken from all patients and study protocols were approved by the institutional review board at the faculty of medicine, Zagazig University.

2.1. Specific Laboratory Investigation

miRNA-155 level is estimated by real time PCR using QIAGEN, miScript, QuantiTect, Rotor-isc (QIAGEN Group) and PAXgene (pre Analytix GmbH).

2.2. Estimation of miRNA-155

2 ml EDTA blood were transferred to 10 ml centrifuge tube to which added an equal volume of balanced salt solution (final volume 4 ml), which was layered onto the Ficoll-Paque media solution (lymphoprepTM, density 1.077 ± 0.001 g/ml, Norway), so mixing the Ficoll-Paque media solution and separating the layer of mononuclear cells. 2 volumes of balanced salt solution were added to the mononuclear cells in the centrifuge tube. Centrifuge at 1200 rpm for 15 min twice. The supernatant was removed. The cell pellet was resuspended in media appropriate for the application.

1) miRNA Extraction:

The miRN easy Mini Kit (QIAGEN®) was used.

The cells were disturbed by adding QIAzol Lysis Reagent®. The cell pellet was loosened by flicking the tube. 700 µl QIAzol Lysis Reagent was added. Vortex was done. 140 µl chloroform (Fisher chemical chloroform, 99.8%, certified AR for analysis, stabilized with amylene, UK) was added to the tube containing the homogenate and then well capped. The tube was shaken vigorously for 15 s, centrifuged for 15 min at 1200 ×g at 4°C in Centurion Scientific®K3cold centrifuge.

700 µl of the sample was added to an RNeasy Mini spin column in a 2 ml collection tube (supplied), Centrifuged at ≥8000 ×g for 15 s at room temperature (25°C). This was repeated twice. 500 µl Buffer RPE was added to the RNeasy Mini spin column, centrifuged for 15 s at 8000 ×g to wash the column. The flow-through was discarded. This was repeated twice.

35 µl RNase-free water was added directly onto the RNeasy Mini spin column membrane, centrifuged for 1 min at 8000 X g to elute the RNA.

2) Reverse-Transcription Reaction:

Reverse-transcription reaction was performed with each sample using ‘miScript II RT Kit’ (Qiagen, Germany).

The reverse-transcription master mix contains all components required for first-strand cDNA synthesis. Template RNA was added to each tube containing reverse-transcription master mix. Mixed gently, briefly centrifuged and then placed on ice. Incubated for 60 min at 37°C. Then, incubated for 5 min at 95°C to inactivate miScript Reverse Transcriptase.

3) Amplification and Detection of cDNA:

Real-time PCR quantification of mature miRNA was done using target specific miScript Primer Assays (forward primer) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany).

4) Data Acquisition and Processing:

Threshold cycle (CT) values were registered for each sample well and were normalized against Snord68. Fold changes of miRNA expression were calculated using $2^{-\Delta\Delta CT}$ method [12].

$$\text{Fold Change} = 2^{-\Delta\Delta CT} \text{ method, } \Delta\Delta CT_T = \Delta CT_{\text{patients}} - \Delta CT_{\text{control}}$$

$$\Delta CT_T (\text{relative expression}) = CT_{\text{miRNA of interest}} - CT_{\text{house keeping RNA}}$$

2.3. Statistical Analysis

The statistical package for social science (SPSS version 16) was used for analysis of the data. For descriptive statistics, the Mean ± SD and Median (range) were used. Comparison between variables was done using the T-test and Chi-square test “and their equivalents” for quantitative and qualitative variables, consequently. The ROC curve was used to detect the cutoff value for miRNA-155. Correlation coefficient was used to assess the correlation between quantitative continuous variables. Survival analysis was done using the Kaplan Meier method and the Log-Rank test was used for comparison between the curves. The level of significance was fixed at 5%, where p value < 0.05 is considered significant.

3. Results

The study included two groups; the control group, which consisted of 40 healthy volunteers and the patient-group, which consisted of 61 patients diagnosed with de novo AML. The two groups were matched for age and sex. The AML-patient group had a significantly higher level of miR-155 expression (mean \pm SD; 7.6 ± 5.16) compared to the control group (mean \pm SD; 2.13 ± 0.63) (Table 1). The cutoff value for miRNA-155 expression was 9.8 “75th percentile value of the AML patients”, where <9.8 was considered low-expression and ≥ 9.8 was considered high-expression (Figure 1). The best fit value of miRNA-155 was detected at ≥ 2.85 with 92.3% sensitivity, 88.5% specificity, 90.4% overall accuracy, area under the curve 0.98 and a 95% CI (0.96 - 0.99) ($p < 0.001$) for discriminating patients from controls (Figure 2). Patients with high miRNA expression had a significantly higher total leukocytic count ($p = 0.002$), bone marrow blasts ($p = 0.006$) and peripheral blood blasts ($p = 0.006$) compared to patients with low miRNA-155 expression, while no difference was found in platelet count, hemoglobin level or age ($p > 0.05$). Patients with poor cytogenetics had a significantly higher level of miRNA-155 expression compared to patients with favorable cytogenetics ($p = 0.007$). The FAB-subtype showed no significant relation to the level of miRNA-155 expression. Patients with low miRNA-155 expression had a significantly higher complete remission rate (38 out of 44 patients, 86.4%) compared to those with high expression (4 out of 17 patients, 23.5%) (Table 2).

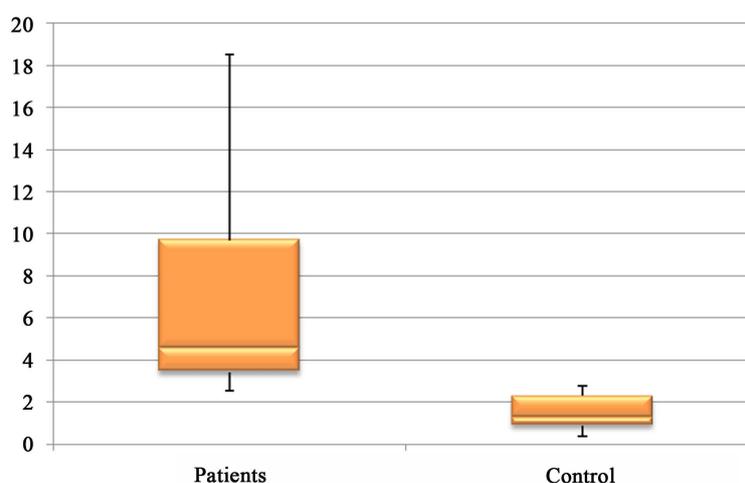


Figure 1. miRNA-155 in the two studied groups.

Table 1. Comparison of miRNA-155 expression level in the two studied groups.

Variable	Patients (n = 61)	Control (n = 40)	T	P
miRNA-155				
Mean \pm SD	7.6 \pm 5.16	2.13 \pm 0.63		
Median	5.35	1.3	6.34	0.012
Range	3.1 - 17.9	0.3 - 3.3		

Table 2. Comparison of clinical and laboratory characteristics with miRNA-155 expression level in AML patients.

Variable	Total (n = 61)	Low miRNA-155 (n = 44)	High miRNA-155 (n = 17)	T	P
Age (years)					
Mean ± SD	45.61 ± 14.52	39.26 ± 12.43	47.73 ± 16.74		
Median	43	30	42		0.58
Range	18 - 55	18 - 53	20 - 55		
TLC (×10³/mm³)					
Mean ± SD	52.2 ± 23.21	32.7 ± 21.54	71.6 ± 29.01		
Median	40	32	65	4.56	0.002
Range	0.9 - 250	0.9 - 125	1.2 - 250		
Hb (gm/dL)					
Mean ± SD	9.2 ± 2.77	9.3 ± 2.37	9.2 ± 1.91		
Median	9.3	9.3	9.1	1.11	0.41
Range	5.5 - 15	5.5 - 15	6.1 - 13.5		
Platelets (×10³/mm³)					
Mean ± SD	83.63 ± 27.42	80.74 ± 25.44	86.52 ± 26.21		
Median	75	72	78	1.13	0.27
Range	5 - 460	5 - 430	7.0 - 460		
PB Blasts (%):					
Mean ± SD	47.5 ± 15.74	38.16 ± 12.39	56.76 ± 18.37		
Median	45	43	60	3.89	0.006
Range	0 - 80	0 - 75	0 - 80		
BM blasts (%):					
Mean ± SD	79.18 ± 13.59	70.83 ± 13.53	87.54 ± 13.81		
Median	70	65	78	3.47	0.008
Range	20 - 98	20 - 98	40 - 98		
FAB subtype					
M2		14 (31.8%)	4 (23.5%)		
M4		13 (29.6%)	5 (29.4%)		0.48
M5		17 (38.6%)	8 (47.1%)		
Cytogenetics					
Favourable		9 (20.5%)	1 (5.9%)		
Intermediate		32 (72.7%)	2 (11.8%)		0.007
Poor		3 (6.8%)	14 (82.3%)		
Response to treatment					
CR	42 (68.9%)	38 (86.4%)	4 (23.5%)		
No CR	19 (31.1%)	6 (13.6%)	13 (76.5%)		0.004

There was a significant positive correlation between miRNA-155 expression level and each of the following; white blood cell count ($r = 0.50$, $p = 0.005$), bone marrow blasts ($r = 0.71$, $p = 0.003$) and peripheral blood blasts ($r = 0.47$, $p = 0.002$) (**Figure 3**), while no significant correlation was found with hemoglobin level, platelet count, LDH or uric acid levels. The disease free survival was

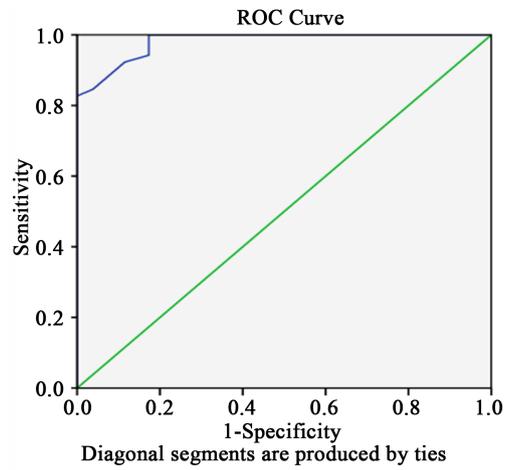
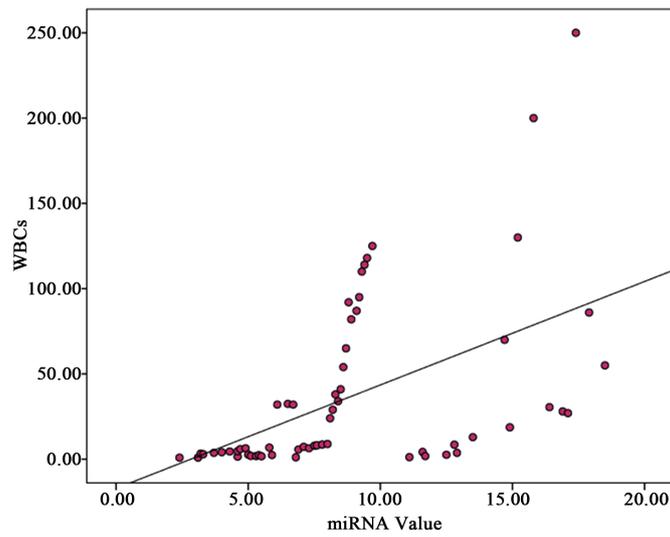
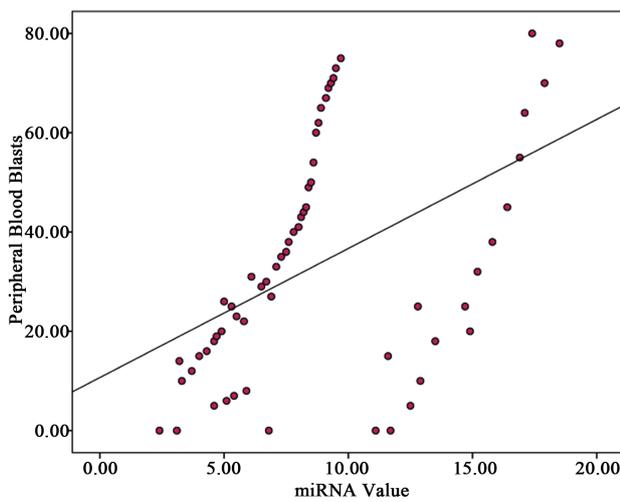


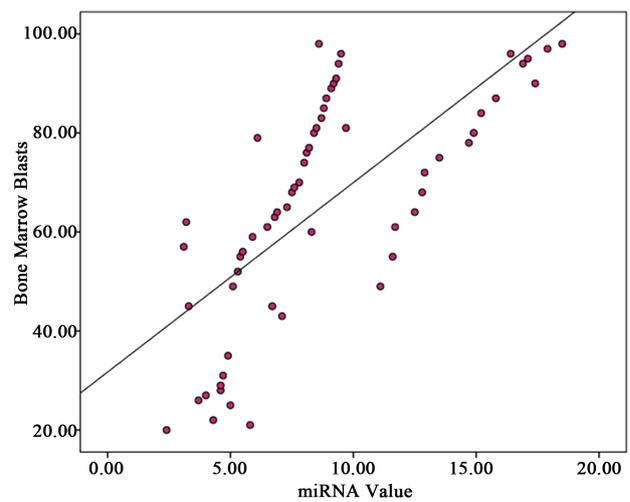
Figure 2. Roc curve for detection of best fit value of miRNA-155.



(a)



(b)



(c)

Figure 3. (a) Correlation between miRNA-155 level and TLC in the patient group; (b) Correlation between miRNA-155 level and PB blasts in the patient group; (c) Correlation between miRNA-155 level and bone marrow blasts in the patient group.

significantly shorter in patients with high miRNA-155 expression compared to those with low expression (the median disease free survival for patients with high-miRNA expression was 12 months, 95% CI (7.4 - 15.5), while the median disease free survival was not reached for patients with low miRNA-155 expression, $p = 0.001$). The overall survival was significantly higher ($p = 0.002$) in patients with low miRNA-155 expression (median overall survival was not reached) compared to patients with high miRNA-155 expression (median overall survival 14.5 months, 95% CI; 10.2 - 17.6) (Figure 4).

4. Discussion

MiRNA-155 expression is an independent prognostic marker for patients with cytogenetically normal acute myeloid leukemia (AML). Patients with higher miRNA-155 expression experience significantly lower rates of complete remission and shorter disease-free and overall survival. Moreover, there are miRNA-155-associated gene expression signature that is enriched for genes involved in survival, proliferation, and inflammation. These findings highlight the potential of miRNA-155 as a prognostic marker and suggest it may also be a therapeutic target (*i.e.*, theranostic molecule) in AML [6].

Although clinical factors have an important role in guiding therapy, cytogenetic changes constitute the single strongest prognostic factor for CR and OS in AML. However, the prognosis of an AML patient cannot yet be estimated accurately. It is therefore important to identify new biomarkers in acute myeloid leukemia patients for the prediction of prognosis, treatment response, detection of relapse, and monitoring for minimal residual disease [13].

MiRNA-155 is a known oncomiR, which is a miRNA that promotes carcinogenic mechanisms in cells usually by repressing tumor suppressor genes. The converse of this is a tumor suppressor miRNA, which is anti-oncogenic in nature by inhibiting expression of cellular oncogenes. In AML, miR-155 is processed from its parent gene miR-155HG, which is located on chromosome 21 [14].

We found that the range of the expression level of miRNA-155 in patients (3.1 -

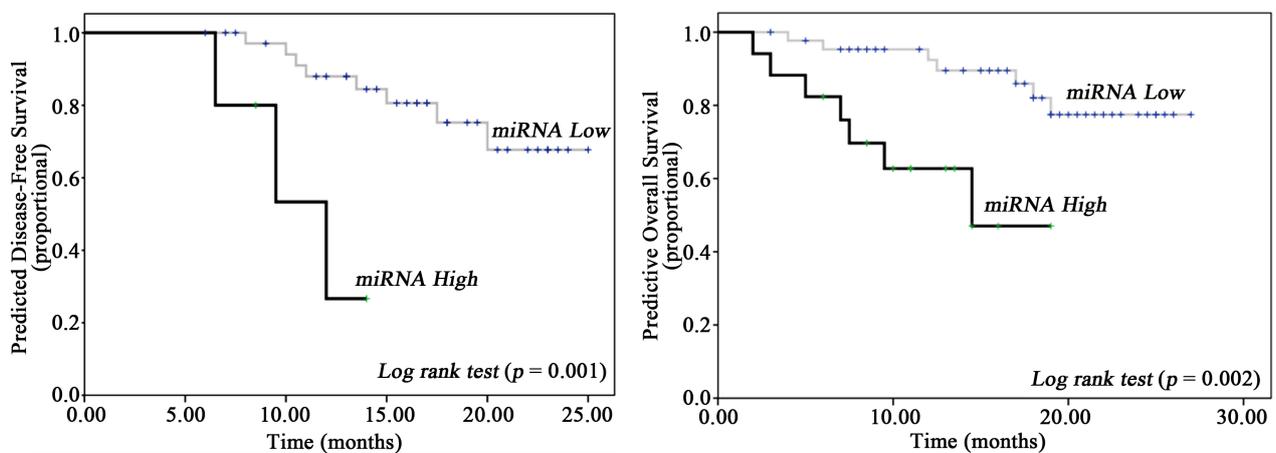


Figure 4. Disease-free and overall survival curves.

17.9) with a median expression level (5.35) and in healthy control (0.3 - 3.3).

In another study done by Ramamurthy *et al.* [15], patients were divided into quartiles (Q1 - Q4) based on individual miR-155 expression levels, where Q1 consisted of those with the lowest miR-155 expression levels and Q4 consisted of patients with the highest miR-155 expression levels and the expression levels of miRNA-155 in the studied groups range from (0.043 - 25.630) with a median expression level of 0.825 this difference may be due to the number and age of patients as they evaluated miR-155 expression in 198 patients with normal karyotype AML (NKAML).

In our study when we compared miRNA-155 expression level in the two studied groups, the fold change was 3.25 and p value < 0.001. The expression level of miR-155 was significantly higher in AML patients than in controls.

Zhi *et al.* [16] also measured miRNA-155 expression level in the serum of 140 adult AML patients and 135 healthy control individuals, they found that there is (4.79) fold change and p value < 0.05. Xu *et al.* [17] found predominant over expression of miR-155 in newly diagnosed 83 AML children with 3.95-fold change, p < 0.0001.

O'Connell *et al.* [18] found that there is 4.5 fold higher when they investigated bone marrow samples from 24 AML patients compared with 6 healthy donors assayed by quantitative PCR.

In our study ROC curve detect 2.85 as best fit value of miRNA-155, with area under the ROC curve (AUC) of 0.98, sensitivity 92.3%, specificity 88.5% and confidence interval (CI) (0.96 - 0.99). The expression level of miR-155 was significantly higher in AML patients than in controls. Also in the study done by Zhi *et al.* [16] miRNA-155 was measured in serum of AML patients and normal controls, ROC curve analysis showed miR-155 AUC, 0.9531 and CI = 0.9259 - 0.9803). These results suggest the potential of miR-155 for discriminating patients from healthy controls.

The 75th percentile value of miRNA-155 expression level for the patient group taken as a prognostic cut off value, which equals 9.8, so patients were divided into those < 9.8 as low miRNA-155 group and ≥ 9.8 as high miRNA-155 expression level group. Xu *et al.* [17] also supported our results when they measured the expression level of miRNA-155 among AML patients they established a prognostic cut off value at 7.8, it is slightly lower than ours which may be due to the age of their patient samples as pediatric patients were included in the study.

Although the presence of lymphadenopathy, hepatomegaly and splenomegaly provides an indirect measurement of leukemic cell burden, none of these clinical variables had any significant association with high miRNA-155 level in our study. Also there is no significant association with other clinical features as presence of fever, pallor, CNS manifestations or bleeding tendency. This comes in concordance with Hu *et al.* [19], who stated that there was no correlation between high miRNA-155 level with any clinical variables.

We found that high miRNA-155 expression level exhibited a statistically significant positive association with TLC with a range of (1.2 - 250), median (65), p

value < 0.001. Also Marcucci *et al.* [6] found that high expression level of miR-155 was significantly associated with high TLC ranged from 1.0 - 450, median (37.9), p value < 0.001 when they investigated 363 adult AML patients. This comes in accordance with Ramamurthy *et al.* [15] and Xu *et al.* [17].

There were high significant differences between high and low miRNA-155 expression levels as regards the percentage of both B.M and peripheral blood blast cells. These results came in agreement with that of Marcucci *et al.* [6] with a p value 0.001 and 0.004 respectively.

Our studied patients were subdivided according to FAB classification into, M2 (13 patients), M4 (16 patients) and M5 (23 patients). We did not find any significant difference between miRNA-155 expression levels and FAB classification (p > 0.05). This go hand in hand with [15] [16] (p > 0.05). In contrast to our results, O'Connell *et al.* [18] identified that AML patients classified as (M4) & (M5) over expressed miRNA-155, but they did not give any explanation for this association.

We found that there was highly significant difference between high and low miRNA-155 expression level groups as regardcytogenetic subtypes with significant increase among high miRNA-155 patients to bad cytogenetics compared to low miRNA-155 patients.

This comes in agreement with the study done by Hu *et al.* [19], reporting that miR-155 expression levels in both poor and moderate prognostic groups were significantly higher compared to favorable prognostic group.

After induction therapy, it was noted that complete remission (CR) rate was significantly lower in the patient group with miRNA-155 level ≥ 9.8 (38.5%) than those with miRNA-155 level < 9.8 (97.4%), p < 0.001 indicating that high level of miRNA-155 level confers a poor outcome. Marcucci *et al.* [6] also supported these results on proving that high miRNA-155 expressers compared with low expressers had a lower CR rate 76% versus 90%. Same findings were reported by Hu *et al.* [19] being 59.09% versus 87.5% (p < 0.05).

Chuang *et al.* [20] found that high miRNA-155 expression level was associated with unfavorable prognosis, including lower complete remission rate.

We found that there was a highly significant increase in the percentage of patients, who had relapsed during follow up together with increased the percentage of dead patients in high miRNA-155 expression level group compared to those with low miRNA-155 expression level (p < 0.01). This came in agreement with [6] [21].

When we followed up our patients we found that Patients with higher miRNA-155 expression levels were associated with shorter disease free survival (DFS) than low miRNA-155 expression level p < 0.001. Also Marcucci *et al.* [6] found that high miR-155 expressers had shorter DFS p < 0.001. This came in agreement with results done by Croce [21].

Therefore, the results of our study, which are in concordance with those reported by several investigators, confirm that miRNA-155 is a prognostic factor for acute myeloid leukemia. Higher levels of miRNA-155 expression were asso-

ciated with lower odds of achieving CR and higher risk for disease relapse or death. [4] [7] [18] established that higher miRNA-155 expression levels were associated with a poorer response to treatment, shorter overall survival (OS) and Disease Free Survival (DFS).

5. Conclusion

The expression level of miR-155 was significantly higher in AML patients than in control group and high miRNA-155 expression level was significantly associated with poor cytogenetics, poor response to therapy and shorter disease free and overall survival conferring a poor outcome. We recommended further studies to work on the usage of anti-miRNAs as a target therapy to target tissues.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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