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The potential role of MiR-328 & MiR-96 as a diagnostic and prognostic biomarker in patients with AML

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Abstract: Background: MiR-328 plays an inhibitory role in the proliferation of cancer cell lines and MiR-96 have a regulatory role. The aim of this work is to study the potential role of MiR-328 & miR-96 as a diagnostic and prognostic biomarker in patients with acute myeloid leukemia (AML). **Materials and Methods:** This study was conducted on 60 persons who were divided into two groups: *Group I:*40 newly diagnosed AML patients were be selected from hematology/oncology unit of Tanta University Hospital and Naser Institute. *Group II:* 20 apparently healthy subjects as a control matched in age and sex with patient's group. Detection of expression levels of MiR-328 and MiR-96 by SYBR Green Real time PCR in both groups. **Results:** Compared with normal controls; The expression of MiR-96 and MiR-328 were significantly down expressed in plasma of newly diagnosed AML patients (p<0.0001). MiR-96 and MiR-328 down regulated were associated with markers of poor risk: high WBCs count, elevated blast count in PB and BM, lower hemoglobin concentration, decrease platelets counts and unfavorable cytogenetics among the studied cases. Overall survival in patients with low MiR-96 and MiR-328 expression were significantly lower than that in those with high MiR-96 and MiR-328 expression. **Conclusion:** Patients with relatively lower levels of MiR-96 and MiR-328 expression had worse outcome in terms of achievement of response and short overall survival.

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Keywords: MiR-96, MiR-328, Acute myeloid leukemia, SYBR Green Real time PCR, Prognosis.

1. Introduction

Acute myeloid leukemia is a life-threatening hematopoietic neoplasm disease characterized by development of a malignant clone of myeloid cells in the bone marrow. Leukemic blasts or immature forms accumulate in the bone marrow, peripheral blood and occasionally in other tissues, resulting in variable reductions in the production of normal blood cells leading to marrow failure [16]. In AML, some factors as age, performance status and history of prior chemotherapy have been associated with the outcome of patients with AML, but the chromosomal abnormalities have been important factor in predicting the risk of relapse [15]. The survival and maturation of hematopoietic cells are affected by deregulation of many signaling pathways with abnormalities in the transcription factors functions presented in normal myeloid differentiation. These pathways could majorly affect AML treatment [14].

MicroRNAs are short, non-protein coding single-stranded RNAs of 21-23 nucleotides in long. Its function regulates gene expression post-transcriptionally by degradation of mRNA or inhibition of protein translation [2]. In acute myeloid leukemia (AML), unique microRNAs signatures are involved in hematopoietic cell differentiation, proliferation, and survival. some of which are used in distinguishing between AML and ALL, and others can differentiate between AML morphological types, cytogenetic and molecular subtypes [20]. They have an impact on prediction of treatment response and clinical outcome, gene expression profiling is believed to be more accurate than molecular mutation detection only and may prove useful tools in the future to help guide therapies [9].

miRNA expression is deregulated in AML by different mechanisms, like: epigenetic changes; copy number alterations; miRNA location in proximity of oncogenic region; altered transcription factors or

oncoproteins; and deregulated miRNA processing finally [23]. Multiple methods have been used for miRNA expression profiling such as miRNA microarrays, Northern blotting, in-situ hybridization, quantitative reverse trascription real-time PCR (qRT-PCR) or deep sequencing [21, 5].

MiR-328 plays an inhibitory role in the proliferation of cancer cell lines known as tumor suppressor; it was found down-regulated in glioblastoma tissues. However, it was up-regulated in non-small cell lung cancer patients [22]. Also, MiR-328 plasma concentrations were significantly elevated in acute myocardial infarction (AMI) patients [8].

MiR-96 is an oncogenic MiRNA, it up-regulated in various types of cancer as in breast cancer [30]. MiR-96 may function as a tumor-suppressing miRNA in renal cancer [28] and pancreatic cancer cell [29]. It acts as a tumor promoting miRNA by increasing the invasive ability of tumors in hepatocellular carcinoma cells [4], non-small cell lung cancer [7], and human bladder urothelial carcinomas [24]. It was shown that MiR-96 expression was positively correlated with liver metastasis in colorectal cancer [27]. Also, it was found to be up-regulated in thyroid papillary cancer [19].

This study was performed to study the plasma levels of MiR-96, and MiR-328 in newly diagnosed AML patients compared to healthy volunteers to evaluate their value in acute myeloid leukemia and to correlate the plasma levels with the clinicopathological features. AML patients were followed after chemotherapy to detect OS and DFS of these miRNAs.

2. Methods

Patients and follow-up

From Mars 2015 to April 2018, 40 patients were diagnosed with de novo AML according to the French–American–British (FAB) criteria at hematology/ oncology unit of Tanta University Hospital and Naser Institute after researched ethical committee approval and informed written parental consent from all participants. Exclusion occurs if patients with other hematological disease, other types of malignancies or the diagnosis is not clear or investigations are incomplete. Patient's group was 18 male and 22 female patients, with a medium age of 36.11 (range 2.5–64) years. 20 apparently healthy subjects served as control group matched in age and sex with Patient's group. None of these controls had previously been diagnosed with any type of malignancy or other benign disease.

All groups were subjected to Full history taking, clinical examination, laying stress on the presence of extramedullary disease (hepatomegaly, bleeding tendency, fever, splenomegaly, and lymphadenopathy), Complete blood picture with examination of peripheral blood smears stained with Leishman stain, Liver function tests, kidney function test, LDH, BM aspiration and examination of BM smears stained with Leishman stain (For patients only), Cytochemical stain for BM smears to confirm the diagnosis by SBB (For patients only), Immunophenotyping, Flourescence in situ hybridization (FISH) analysis (For patients only).

The median leukocyte count at diagnosis was 53, $44*10^3$/ L (range 1.5 – $199*10^3$/ L). According to the FAB classification, three patients had AML M0, five had M1, six had M2, seven had M3, eleven had M4, four had M5, two had M6, and two had M7. All patients were subjected to cytogenetic classification according to karyotyping and detection of common fusion genes, including, PML-RARά, t (8;21), +8, -7, inv (16), and BCR/ABL. Clinical characteristics of the patients with AML are summarized in Table 1. All patients received chemotherapy and were followed up in duration about 2 years after treatment.

AML complete remission (CR) was defined as a normocellular BM containing less than 5 % blasts and showing evidence of normal maturation of other marrow elements; a neutrophil count of 1 × 109/L and a platelet count of 100 × 109/L. 21 patients achieved CR, 4 cases failed to achieve complete remission (refractory), and 15 cases relapsed. Relapse was defined as reinfilteration of the bone marrow by 5% or more leukemic blasts or proven leukemic blasts at any site. Overall survival (OS) is the period from the date of diagnosis to the date of last follow up or death. The patients were followed for two year and the probability of overall survival was determined. Disease- free survival (DFS) is the duration for the start of complete remission to the time of relapse from CR. DFS applies only to patients in complete response.

Plasma collection and RNA extraction

Blood samples were collected in EDTA tubes and processed within 1 h of collection. plasma was isolated from all blood samples using a centrifugation for 10 min at 3000 g. The supernatant was transferred to RNase/DNase free tubes and stored at −80 °C.

Syn-cel-miR-39 (Synthetic C. elegans) miScript miRNA Mimic *(cat. no. MSY0000010)* miRNA can be added to samples to control for variations during the preparation of total RNA and subsequent steps. RNA extraction was done manually using miRNeasy Mini Kit (Qiagen company, Hilden, Germany) (Catalog No. 217004).

RT-PCR

All extracted total RNA from all patients and controls were converted to cDNA by reverse transcription using miScript Π Reverse Transcription

Kits (Qiagen company, Hilden, Germany) (cat. No. 218160, 218161).

Briefly, the reverse transcription reaction was performed in 20 µL mixture containing 4 µL 5 × miScript HiFlex Buffer, 2 µL 10 × miScript Nucleics Mix, 2 µL RNase Free water and 12 µL Template RNA. The 20-µl reaction volumes were incubated at 37°C for 60 min, 95°C for 5 min, and then held at 4°C or stored at −20°C.

Quantitative real-time PCR

Detection the expression levels of the target (MiR-328 & MiR-96), endogenous control (SNORD69) and internal control (miR-39) by SYBR Green Real time PCR by using 7500 device and miRNeasy Mini Kit (Qiagen company, Hilden, Germany) (Catalog No. 218073, 218075, 218076). The 25-µl PCR mixture included 12.5 µl Quantitect Sybr green PCR master mix, 2.5 µl 10x miScript Universal Primer, 2.5 µl 10x miScript Primer Assay, 3.5 µl RNase Free water and 4 µl cDNA. PCR program conditions were 95 °C for 15 min, followed by 45 cycles of 94 °C for 315 s, 55 °C for 30 s and 70°C for 30 s.

Resultant miRNA levels were normalized using Syn-cel-miR-39miScript miRNA Mimic. The relative expression level of MiR-328 & MiR -96 was calculated by the equation of 2-ΔCt (ΔCt=Ct MiR-328 or MiR -96 - Ct miR-39miScript miRNA [12]. The fold changes in MiR-328 or MiR -96 were calculated using the 2-ΔΔCt method [18].

3. Results

MiR-328 & miR-96 were down regulated in AML patients

The MiR-328 & MiR-96 expression levels were detected in plasma samples from patients with AML and healthy controls by qRT-PCR. As shown in (Fig. 1) plasma concentration of MiR-328 was markedly down regulated in AML patients (median expression value 32.89, range: 28.8-41.34) relative to those in healthy controls (median expression value 68.44, range: 57.87-73.8; $P < 0.001$). In addition, plasma concentration of MiR-96 was down regulated in AML patients (median expression value 48.42, range: 35.19-52.68) relative to those in healthy controls (median expression value 89.46, range: 63.6 -93.63; $P < 0.001$).

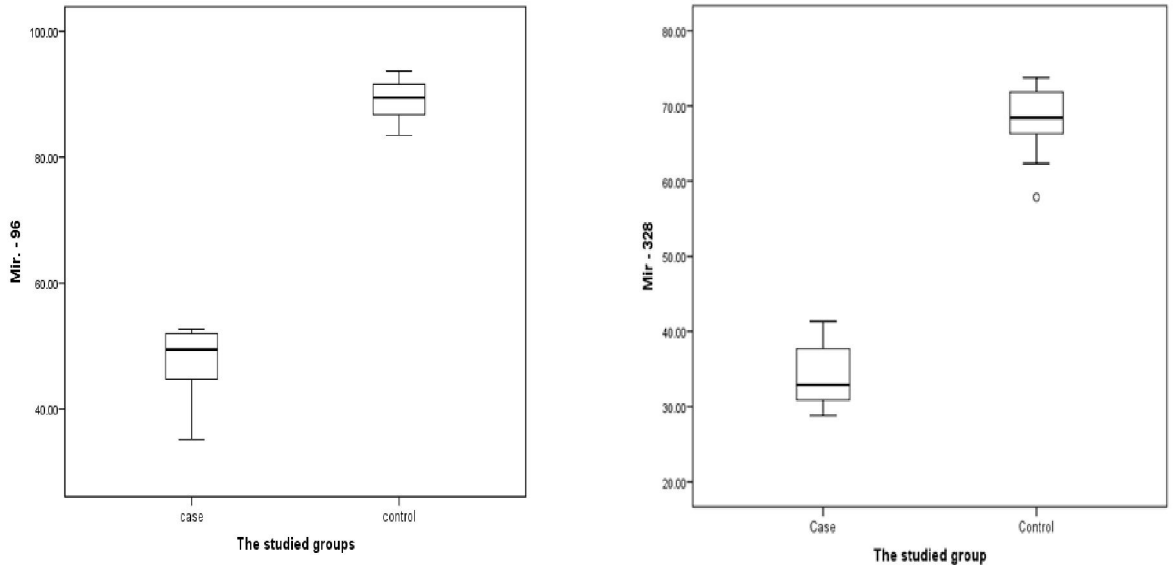

Figure 1: Comparison of Mir-96 & Mir-328 expression among cases and control.

AML patients expressing MiR-328 at levels less than the median (54.16) were assigned to the low-expression group (mean expression value 9.47), and those samples with expression equal to or above the median value were assigned to the high-expression group (mean expression value 17.43). AML patients expressing MiR-96 at levels less than the median (57.5) were assigned to the low-expression group (mean expression value 9.26), and those samples with expression equal to or above the median value were assigned to the high-expression group (mean expression value 18.28).

Correlation of MiR-96 & MiR-328 expression with clinical characteristics of AML

Lower levels of MiR-96 were associated with a higher white blood cell count, BM blast count, and lower hemoglobin and platelet count. The intermediate and unfavorable cytogenetics of AML had significant association with Mir-96. Study of Mir-96 in relation to response of therapy on day 28, Patients who did not

achieve CR (relapsed & refractory) had significantly association with Mir-96 than those who were in CR after initial chemotherapy, (p<0.001) Moreover, we failed to correlate the expression levels of MiR-96 with other clinical parameters including sex, age, clinical data and FAB subtype. Lower levels of miR-328 were associated with a higher white blood cell count, BM blast count, and lower hemoglobin and platelet count. The intermediate and unfavorable cytogenetics of AML had significant association with Mir-328. Study of Mir-328 in relation to response of therapy on day 28, Patients who did not achieve CR (relapsed & refractory) had significantly association with Mir-328 than those who were in CR after initial chemotherapy, (p<0.001) Moreover, we failed to correlate the expression levels of Mir-328 with other clinical parameters including sex, age, clinical data and FAB subtype.

Table 1: Clinical characteristics among newly diagnosed AML & controls

	The studied groups		U test	P value
	Cases N = 40	Control N = 20		
Age Mean ±SD Range	 36.11±16.55 2.5 – 64	 32.1±18.91 3 – 67	0.74	0.46
TLC (10^3/ L) Mean ±SD Range	 53.48±54.93 1.5 – 199	 8.66±2.50 3.9 – 12.5	3.04	<0.001*
Hb (gm/ dl) Mean ±SD Range	 8.17±1.78 4.2 – 11.7	 12.3±0.75 11.5 – 14.0	9.64	<0.001*
Platelets (10^3/ L) Mean ±SD Range	 52.25 ±49.25 6 – 246	 289.9±95.86 150 – 440	5.45	<0.001*
RBCs (10/ L) Mean ±SD Range	 2.98±0.92 1.5 – 4.07	 4.14±0.65 3.5 – 5	3.85	<0.001*
PB. blasts (%) Mean ±SD Range	 48.43±29.58 14 – 92	 0.0±0.0 0 – 0	5.49	<0.001*
BM. blasts (%) Mean ±SD Range	 65.92±27.58 4 – 95	 0.0±0.0 0 – 0	6.26	<0.001*

U = Mann Whitney U test was used for comparison. * Statistically significant

Association of Mir-96 & Mir-328 expression with clinical outcomes of AML

The Kaplan–Meier curves for newly diagnosed patients after received chemotherapy showed that mean overall survival was 14 months among the studied cases **fig (2)**

Low Mir-96 was significantly associated with shorter overall survival compared to high Mir-96 in AML group **fig (3)**.

Low Mir-328 was significantly associated with shorter overall survival compared to high Mir-328 in AML group. **Fig (4)**.

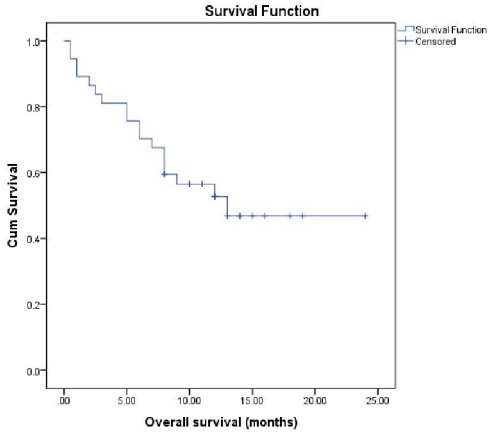

Figure (2): Mean survival among the studied cases

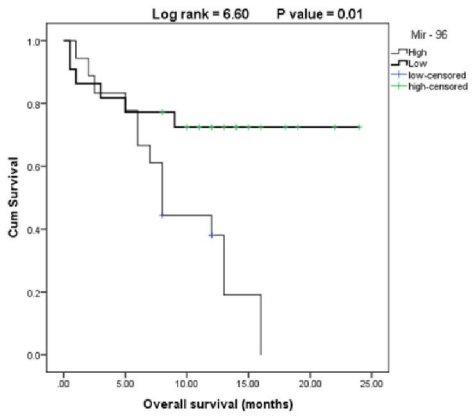

Figure (3): Mean overall survival in relation to low and high Mir-96 among the studied cases

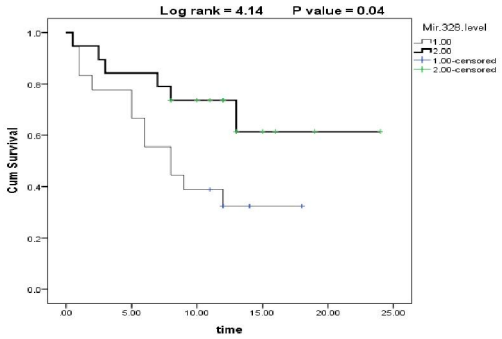

Figure (4): Mean overall survival in relation to low and high Mir-328 among the studied cases

4. Discussion:

In acute myeloid leukemia (AML), microRNAs are involved in hematopoietic cell differentiation, proliferation, and survival. They have an impact on treatment response and outcome. Different microRNA expression profiles are seen in various cytogenetic groups of AMLs [17].

MiRNA expression is frequently deregulated in AML by different mechanisms, like: (i) copy number alterations; (ii) epigenetic changes; (iii) miRNA location in proximity of oncogenic genomic region due to chromosomal translocation or overexpression of protein-coding gene; (iv) aberrant targeting of miRNA promoter regions by altered transcription factors or oncoproteins; and finally (v) deregulated miRNA processing [23].

MiR-96 and MiR-328 were found to be significantly down-regulated in plasma of AML patients with median fold change 49.42 and 32.89 respectively compared to the healthy control group. In 2014, the results of a study done in China by ***Zhao et al.,*** [31] has revealed that plasma MiR-96 was significantly downregulated in newly diagnosed AML patients compared to healthy controls (p<0.0001). Later on, in 2015, ***Li et al.,*** [13] have found in a study done on a Chinese population that miR-328 has showed significantly lower expression in the plasma of newly diagnosed AML patients than in normal controls (p<0.0001).

MiR-96, together with miR-182 and miR-183, belongs to the miR-183-96-182 cluster, which has been demonstrated to play important roles in tumorigenesis and tumor progression [25]**.**

Lin et al., (2010) [11] reported that circulating MiR-96 expression was significantly higher in breast cancer cells; overexpression of MiR-96 induces cell proliferation and growth. ***Chen et al., (2012)*** [4] found that plasma MiR-96 concentrations were significantly elevated in hepatoma.

By contrast, accumulating studies have demonstrated that tumor suppressive roles of MiR-96 are found in other types of cancer. MiR-96 levels are markedly decreased in pancreatic cancer [29].

MiR-328 is proposed as a suppressor gene by targeting proto-oncogene serine/threonine-protein kinase PIM1 and translational regulator protein hnRNP E2 [1]. MiR-328 could also inhibit epithelial mesenchymal transition (EMT) via targeting CD44 [3]. These findings indicate that miR-328 plays a direct role in the modulation of cancer progression and may be useful as a novel prognostic or progression marker for cancer. ***Eiring et al., (2010)*** [6] reported that MiR-328 was down-regulated in chronic myelogenous leukemia blasts, and low expression of MiR-328 in CML is associated with progression to the blast crisis phase of the disease. ***Wu et al., (2012)*** [26] observed that MiR-328 expression is decreased in high-grade gliomas and is associated with worse survival in primary glioblastoma. In contrast ***Ulivi et al., (2013)*** [22] reported that circulating MiR-328 expression was significantly higher in non-small cell lung cancer (NSCLC) patients than in healthy donors. ***Wang et al., (2014)*** [5] found that plasma MiR-328 concentrations were significantly elevated in acute myocardial infarction (AMI) patients compared to those control subjects.

The present study showed that there was a non-significant statistical difference regarding age and gender distribution among the studied groups. These results are in agreement with ***Zhao et al., (2014)*** and ***Liu et al., (2015)*** [23, 13]**.**

In the present study, down regulation of miR-96 and miR-328 was negatively correlated with WBCs count and blast count in PB and BM. Also, positively correlated with hemoglobin concentration and platelets counts but no significant correlations were found with CRP and LDH which agreement with ***Zhao et al., (2014)*** [23] reported that down regulation of MiR-96 was associated with a higher white blood cell count and bone marrow blast count, and lower hemoglobin and platelet count. Similarly, in a study that was

conducted by ***Li et al., (2015)*** [13] it was found that down regulation of MiR-328 in AML patients was significantly associated with a higher WBC count and blast count in BM, and lower HGB and platelets counts.

In the present study low MiR-96 and low MiR-328 expression was associated with intermediate cytogenetic (normal karyotype for sex, +8) and unfavorable cytogenetic (BCR/ABL, -7) of AML**.** Interestingly MiR-96 and MiR-328 expression did not associate with treatment outcome in the favorable cytogenetics [t (8;21), inv (16), PML-RARά] of AML group indicating that Low MiR-96 and MiR-328 expression did not seem to affect response to treatment in this category.

Our data suggest that MiR-96 and MiR-328 expression is important in determining the patient's response to treatment. Patients who did not achieve CR (relapsed & refractory) had significant association with low expression of both MiR-96 and MiR-328 than those who were in CR after initial chemotherapy.

These data were also obtained by ***Zhao et al., (2014)*** [23] who reported that down regulation of MiR-96 was associated with unfavorable cytogenetic risks so, expression of MiR-96 has an important value in AML prognosis classification. ***Li et al., (2015)*** [13] found that circulating MiR-328 act as a suppressor gene in the development of AML, and have an adverse effect on prognosis of AML patients.

Conclusions:

MiR-96 and miR-328 were down expressed in plasma of AML patients in the present study compared to control samples. MiR-96 and miR-328 down regulated were associated in our patients with markers of poor risk: high WBCs count, elevated blast count in PB and BM, lower hemoglobin concentration, decrease platelets counts and unfavorable cytogenetics among the studied cases. Patients with relatively lower levels of miR-96 and miR-328 expression had worse outcome in terms of achievement of response and short overall survival.

Recommendation:

Further study on a large scale is recommended to explore more the utility of MiR-96 and MiR-328 in AML patients.

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