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Quantification of microRNA-181b in Egyptian Acute Myeloid Leukemia Patients and its Relation to Prognosis

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Abstract
Unique micro RNA (miRNA) expression signatures are aberrantly expressed in acute myeloid leukemia (AML). Certain miRNA signatures have been associated with prognosis of AML, one of these is miR-181b. We aimed to quantify miR-181b in AML Egyptian adult patients and evaluate its prognostic significance in those patients. Eighty newly diagnosed AML patients with 40 age and sex matching healthy volunteers as a control group were studied for the expression of miR-181b by stem-loop quantitative reverse-transcription polymerase chain reaction (qRT- PCR).miR-181b expression level was higher in patients' group than controls (p=0.013). Patients with miR-181b ≥ 6.0 were associated with favorable molecular abnormalities and most of them achieved complete remission (CR), whereas most of patients with miR-181b< 6.0 were associated with unfavorable ones and failed to achieve CR. Patients with miR- 181b values ≥ 6 showed significantly higher mean survival than patients with values <6 (35±0.7, 14.6±2 month respectively, p<0.001).Low value of miRNA181b was identified as an independent predictor of bad prognosis. High expression level of miR-181b is detected in Egyptian AML adult patients identifying a distinct group with favorable prognosis
Keywords: *miR-181b, acute myeloid leukemia, prognosis*

Introduction

Micro-ribonucleic acids (miRNAs) are small non-coding RNAs of 19–24 nucleotides in length that regulate gene expression by base pairing with a target gene's mRNA, leading to degradation and/or translational repression of that gene. miRNAs have been implicated in many biological events, and their deregulation is associated with leukemogenesis [1], also with heart diseases, nervous system diseases, obesity and viral infections [2].

It is known that miRNA-181b (miR-181b) is an oncogenic miRNA over expressed in several cancers, such as colorectal cancer, acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML).miR-181b

was found to contribute to AML cell proliferation and likely inhibition of cell apoptosis. This occurs through depression of the expression of mitogen- activated protein kinase 10 (MLK2), whose product is a member of mitogen-activated protein kinase (MAPK) pathway which regulates diverse processes, ranging from proliferation and differentiation to apoptosis [3].

Currently, the disease-free survival of AML is <50% after intensive chemotherapy. Thus, more effective regimens are needed to prolong the duration of remission and the long-term survival. Studies report that miR-181b is highly expressed in AML and represents a promising therapeutic target for improving the efficacy of AML recovery [3]. Consequently, miRNA profiling has become the standard discovery tool to identify miRNAs implicated in diseases or other cellular processes. Complementary DNA (cDNA) microarrays and real time polymerase chain reaction (RT-PCR) are increasingly popular technologies used to profile miRNAs [4].

In this study we aimed to relatively quantify miR-181b in Egyptian AML patients, to identify its role as a prognostic factor in AML, and to elucidate the relation between its expression level and patients' outcome together with standard prognostic factors in AML.

Patients and Methods

This prospective study was carried out on 80 de novo AML patients attending the Hematology Oncology Unit of Ain -Shams University Hospitals during the period from January 2012 to January 2015. Their age ranged from 14 to 73 years old, with mean age of 40.2 ± 17.7 years. Forty six (57.5 %) of them were males and 34 (42.5 %) were females, with male to female ratio of 1.3:1.Fourty healthy volunteers matching in age and sex were studied as a control group. Their age ranged from 16 to 69 years old, with mean age of 38.8 ± 15.2 years. Twenty (50%) were males and 20 (50%) were females with male to female ratio of 1:1.The procedures applied in this study were approved by the Ethical Committee of Human Experimentation of Ain Shams University and are in accordance with the Helsinki Declaration of 1975.

All patients were subjected to full history taking, thorough clinical examination laying stress on the presence of extramedullary disease (hepatomegaly, splenomegaly, and lymphadenopathy) together with laboratory investigations including complete blood count (CBC) using LH 750 analyzer, examination of Leishman-stained peripheral blood (PB) and/or bone marrow (BM) smears, examination of Myeloperoxidase-stained PB and/or BM smears, Immunophenotypingon BM/PB samples by whole blood lysis performed by EPICS XL Flow Cytometer, Coulter, USA using the routine panel of monoclonal antibodies for acute leukemia [5], as well as conventional cytogenetic analysis (CCA) and molecular cytogenetics using fluorescence in situ hybridization (FISH) probes for CCA failed cases [6] on PB or BM samples. FISH analysis of PB/BM samples using routine panel of probes for AML; ETO/AML1 for t(8;21), PML/RARa for t(15;17), CBFB/MYH11 for Inversion 16, MLL rearrangement for 11q23 rearrangements, BCR/ABL for t(9;22), EV11 rearrangement for 3(q26). Finally detection and quantification of miR-181b expression levels was done by RT- PCR using Light Cycler probe design soft ware (Roche Diagnostics, GmbH, Germany).

Diagnostic criteria

Diagnosis of AML was based on the presence of 20% or more blasts in BM [7], together with myelperoxidase positive staining and immunophenotyping pattern.

Assessment of patients' therapeutic response was done at day 28 following the initial diagnosis and induction of therapy and by assessment of overall survival. Patients were re-evaluated as regards achieving remission or not both clinically and laboratory; by CBC and BM re-examination. Cytogenetic risk classification were defined according to European Leukemia Net (ELN) [8].

Treatment regimens

Therapeutic responses were defined according to the criteria developed by the International Working Group [9]. All AML patients received treatment according to the ESMO Clinical Practice Guidelines [10].

Methods

Sample collection

Peripheral blood and BM samples were collected on ethylene diamine tetra-acetic acid (EDTA) (1.5 mg⁄ mL) for morphological, cytochemical, and immunophenotypic analyses and on lithium heparin for cytogenetic and molecular analyses.

Nucleic acid isolation and stem-loop quantitative reverse-transcription PCR (qRT- PCR) assay

qRT– PCR amplification was done using TaqMan® MiRNA Reverse Transcription Kit (P/N 4366596) and TaqMan® MiRNA primers and hybridization probes for miR-181b and RNU(P/N 4427975) (Applied Biosystems) and TaqMan® miRNA controls in combination with the TaqMan Universal PCR Master Mix II (Applied Biosystems). The total RNA was extracted from PB/BM samples using a Ribo Pure™- Blood Kit (Applied Biosystems, Ambion Cat#AM1928) according to the manufacturer's protocol.

Complementary deoxyribonucleic acid was synthesized using TaqMan gene expression assay kit (Applied Biosystems) and stored at -20°C until use. PCR products were then synthesized from cDNA samples using the TaqMan Universal PCR Master Mix (Applied Biosystems). Each PCR contained all the necessary reagents and 50 ng of cDNA in a final volume of 20 µL. The reaction protocol involved heating for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). Analysis of data was carried out using the Startagene Mx3000P software (Startagene Inc., CA, USA). miR-181b expression levels in unknown samples were calculated by relative quantification using the 2-ΔΔCT Method [11] as a ratio of miR-181b to glyceraldehyde-3-phosphate dehydrogenase. A negative control without a template was included in each assay. The cycle threshold (Ct) values of the samples were compared with RNA obtained from a healthy individual to be used as a calibrator [12].

Cytogenetics

Cytogenetics were performed according to Schoch et al., 2002 [13]. In all cases, 15–20 metaphases were analyzed and classified according to the International System for Human Cytogenetic Nomenclature [14].

Statistical Analysis

Statistical analysis of the data was performed by using IBM SPSS 22.0 software package under Windows 8.1 operating system. Categorical data parameters were presented in the form of frequency and analyzed for group differences by using chi square test (X^2 value) or Fisher exact test according to nature of the data. Continuous data parameters were analyzed for normality by using Shapiro-Wilk test. Comparative analysis was performed by using Mann-Whitney U test (Z value) for two independent samples. The receiver operating characteristic (ROC) curve was used to determine the best cut-off values of miR-181b that accurately discriminate AML patients into complete remission (CR) and incomplete remission or non-remission (IR/NR).

Kaplan-Meier curve for survival distributions of studied groups was plotted. Log rank pair-wise comparison (X^2 value) was run to determine the difference in survival between studied groups. A logistic regression was performed to ascertain the effects of miRNA 181b as independent variable on the likelihood that participants have bad disease outcome.

Probability level (P value) was assumed significant if less than 0.05 and highly significant if P value was less than 0.001. P value was considered non-significant if greater than or equal to 0.05.

Results

The demographic, clinical and laboratory characeteristics of AML patients' group are illustrated in Table 1. The mean of miR-181b expression level was higher in patients' group compared to controls (14.76±18.21, 0.92±0.22 ,p=0.013). Follow up of the AML patients was done to identify their therapeutic response. Fifty two patients 52/80 (65%) achieved CR, while 28/80 (35%) exhibited IR/NR. Subsequently patients were subdivided into two groups; (CR) versus (IR/NR) (Table 2).

A receiver operating characteristic curve (ROC) curve for relative quantification of miR-181b was done and revealed that a cut off value of 6.0 was the best value that accurately discriminated the AML patients into CR and NR, in addition it showed 85.7% sensitivity and 96.1% specificity (Figure 1). Regarding relation to therapeutic response, 50/52 (96.1%) of the patients who achieved CR had miR-181b expression level ≥ 6.0, while 24/28 (85.7%) of the patients who failed to achieve CR had miR-181b expression level< 6.0 (p< 0.001). In addition, patients with miR-181b ≥ 6.0 were significantly associated with favorable molecular abnormalities, whereas patients with miR-181b< 6.0 were significantly associated with unfavorable molecular abnormalities (p< 0.001) (Table 2).Multivariate analysis using logistic regression was performed and Wald chi square test that used to determine statistical significance for each of the independent variables was statistically significant for miRNA 181b (X2=18.867, p<0.001) indicating that low value of miRNA 181b is an independent predictor of bad prognosis.

Survival analysis

Overall mean survival of studied patients was 30 month (95% CI, 27.7 to 33.0). Patients with miR- 181b with values ≥ 6 had mean survival of 35 month (95% CI, 33.5 to 36.4); while patients with values <6 had mean survival of 14.6 month (95% CI, 10.7 to 18.5). Log rank test for equality of survival distribution of both studied groups showed highly significant difference (x^2=26.97; p<0.001) (Figure 2).

Table 1: Characteristics of AML patients

Parameter	**Patients** n=80		Mean± SD
	n	**%**	
Age/Years			
≥60	14	17.5	40.28±17.72
<60	66	82.5	
Sex			
Male	46	57.5	Ratio 1.3:1
Female	34	42.5	
Hepatomegaly			
+ve	22	27.5	-
- ve	58	72.5	-
Splenomegaly			
+ve	30	37.5	-
- ve	50	62.5	-
Lymphadenopathy			
+ve	26	32.5	-
- ve	54	67.5	-
TLC x 10^9/L			
≥50	28	35	58.97±76.13
<50	52	65	
Hb g/l			
≥10	14	17.5	7.96±2.25

<10	66	82.5	
Plt x10^9/L ≥100 <100	 20 60	 25 75	66.88±55.04
PB Blasts	-	-	46.15±32.24
BM Blasts	-	-	58±22.20
Immunophenotype (FABsubtype) M0/M1 M2 M3 M4 M6	 22 28 10 18 2	 27.5 35 12.5 22.5 2.5	 - -
Conventional karyotyping Favorable Intermediate Unfavorable	 26 41 7	 32.5 51.25 8.75	
FISH Favorable Intermediate Unfavorable	 25 43 12	 31.25 53.75 15	

TLC=Total leucocytic count, Hb=Hemoglobin Plt: platelets, PB: peripheral blood, BM: bone marrow

Table (2): Difference between low and high miR-181b expression groups regarding patients' characteristics and therapeutic response

		miR- 181b					
		≥6 n=54		<6 n=26		X^2/Z	P
Age		n	%	n	%		
≥60	66	46	69.7	20	30.3	-	0.366
<60	14	8	57.1	6	42.9		
Sex							
Male	46	30	65.2	16	34.8	0.257	0.612
Female	34	24	70.6	10	29.4		
TLC							
<50	52	38	73.1	14	26.9	2.106	0.147
≥50	28	16	57.1	12	42.9		
Hb							
≥10	14	10	71.4	4	28.6	-	1.000
<10	66	44	66.7	22	33.3		
PLT							
≥100	20	14	70.0	6	30.0	0.076	0.783
<100	60	40	66.7	20	33.3		
PB blasts		37 (14-70)		57 (21.5-91.25)		1.481z	0.139
PB blasts		68 (50-76)		60 (35-71.25)		1.586z	0.113
FISH							

Favorable	25	23	92.0	2	8.0	26.090	<0.001
Intermediate	43	30	69.8	13	30.2		
Unfavorable	12	1	8.3	11	91.7		
Response							
CR	52	50	96.2	2	3.8	50.505	<0.001
IR/NR	28	4	14.3	24	85.7		
Auer rods							
Negative	54	34	63.0	20	37.0	1.559	0.212
Positive	26	20	76.9	6	23.1		

TLC=Total leucocytic count, Hb=Hemoglobin, Plt: platelet, PB: peripheral blood, BM: bone marrow, CR: complete remission, IR/NR: incomplete remission or non remission

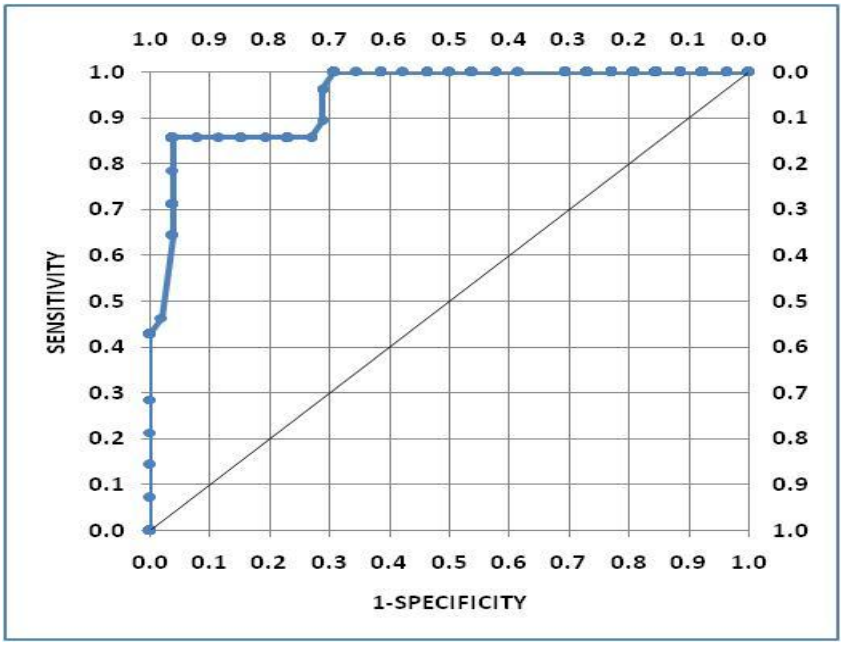

Figure (1): Determination of cut-off value for miRNA181-b by ROC curve

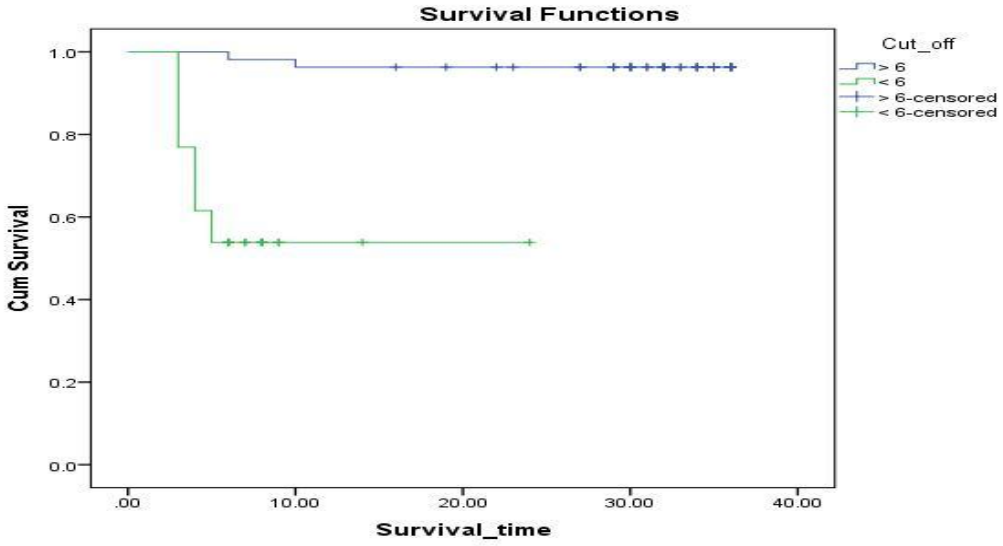

Figure (2): Kaplan-Meier overall survival time for AML patients according to microRNA181b over expression

Discussion

miR-181b expression level was significantly increased in our Egyptian AML patients compared to control group which may denote a pathogenic role of miR-181b in the leukemogenic process.

Previously, an English study, compared leukemia BM samples with BM from healthy donors and high lightened the differential expression of a number of miRNAs potentially involved in the oncogenic process. miR-181b was identified as one of the seventeen miRNAs upregulated in AML in their research [15].

Additionally, other investigators showed that miR-181b was highly expressed in AML patients when compared to healthy controls. Their results suggest that miR-181b may play a role in AML leukemogenesis by different etiological mechanisms [1], [3]. miR-181b has already reported high expression in a variety of hematological malignancies. Moreover, it was reported that miR-181b contributes to AML cell proliferation and likely inhibition of cell apoptosis [15].

Interestingly, it was stated that miR-181b acts through depression of mitogen - activated protein kinase pathway (MAPK pathway) which regulates diverse processes, ranging from proliferation and differentiation to apoptosis [3]. Moreover, a previous study identified genes regulated by miR-181b whose altered expression contributed to leukemogenesis. Many of these genes encode proteins involved in pathways of innate immunity mediated by Toll-like receptors (TLR4, TLR2, TLR8) and interleukin 1B and caspase-1 (IL1B, CARD 8, 12, 15, CASP1). Activation of Toll-like receptors induces production of inflammatory cytokines which promote the survival and proliferation of AML blasts. These genes are down regulated by miR-181 family [16].

In the present study high miR-181b expression levels (≥6) were consistently associated with better prognosis and favorable cytogenetics. Six patients had MLL gene rearrangement showed miRNA 181b< 6.0 and failed to achieve CR. While twelve out of sixteen patients with t (15,17) showed miRNA 181b≥ 6.0 and achieved CR. Similarly, distinct miRNA expression profiles were identified in AML with common translocations. One of these was miR-181b which was significantly down regulated in MLL rearrangements; which has unfavorable prognosis, and was upregulated in t(15; 17), which has favorable prognosis [17]. These data were confirmed later by a review literature by Havelange et al. in 2009 [18].

Additionally, molecular studies on cytogenetically normal AML patients (CN-AML) showed that CEBPA mutations, which carry favorable prognostic value in CN-AML, have been associated with an upregulation of members of miR-181 family [16]. They integrated gene expression with miRNA expression profile and observed a functional relationship between them. They concluded that miRNA deregulation results in altered genetic expression and contributes to leukemogenesis in molecular high-risk CN-AML [1],[16]. Another interesting evidence supporting this relation was introduced by Hollink et al in 2011[19], who found that translocations involving chromosome 11q15 (nucleoporin 98kD) are associated with HOX gene expression and poor prognosis. miR-181b alternatively down regulates Hox genes. Our results for the patients harbouring chromosome 11 rearrangement showed miRNA 181b< 6.0 which explains their adverse therapeutic response.

In this work, patients with miR- 181b with values ≥ 6 had a longer mean survival than patients with values <6. In accordance, Marcucci et al. in 2011 [16], related a miRNA expression signature with prognosis in CN-AML patients. They found that increased expression of miR-181b is associated with decreased failure to achieve CR, relapse, or death. Moreover, it was reported before in a mini review article that miRNA signature; including miR-181b and miR-181a, correlated with event free survival in young AML patients with high risk molecular risk (FLT3-ITD and/or NPM1 [20].

Conclusion

Finally, the foregoing discussion suggests that high expression level of miR-181b may identify distinct group of Egyptian Acute Myeloid Leukemia patients with favorable prognosis. It has a remarkable association with cytogenetics as prognostic predictors of outcome and a striking effect on the therapeutic response. Being an independent factor of poor prognosiosits low expression may complement and enhance our current knowledge about AML, which is relatively easy to measure for molecular risk assessment of individual patients at diagnosis.

Conflict of Interest

The authors report no conflicts of interest.

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