

Expression Signature of MicroRNA-155 and its Association with Response to Treatment within Different Subtypes of B-Cell Malignancies

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Abstract

Objectives: Emerging evidence suggests that microRNAs could serve as non-invasive biomarkers for cancer patients. However, they are mostly not fully investigated as biomarkers in the classification, progression and prognosis of different cancers. Our study was designed to explore the microRNA-155 (miR-155) expression levels in peripheral blood mononuclear cells (PBMCs) in patients with various subtypes of B-cell malignancies. Also, we aimed to correlate between miR-155 expression levels and the diverse clinico-pathologic features as well as the prognostic fate of these patients after treatment completion.

Subjects and methods: Using whole blood samples from 53 patients with B-cell malignancies and 15 apparently healthy subjects, miR-155 was extracted and profiled by quantitative real-time PCR (RT-qPCR). The B-cell malignancies patients were including 22 diffuse large B-cell lymphoma (DLBCL), 15 chronic lymphocytic leukemia (CLL), 9 follicular lymphoma patients (FL) and 7 subjects with burkitt's lymphoma (BL). The samples were withdrawn before starting chemotherapy in addition to after completing their therapeutic courses by 6 months. Subsequently, the patients were further sub-grouped into those with partial remission, complete remission, resistant disease and relapse.

Results: We found that miR-155 expression levels differentiate lymphoma entities from normal subjects ($p \leq 0.001$) and its expression fold changes distinguish B-cell lymphoma subtypes from each other's ($p < 0.05$). In addition, miR-155 was significantly correlated with age and LDH levels. The receiver operating curve (ROC) was employed to identify the diagnostic outcomes of miR-155 in discriminating B-cell malignancies entities from each other's (AUC=0.957 for DLBCL vs. CLL+FL and AUC=1.000 for CLL vs. FL). Otherwise, when BL was versus healthy controls the AUC was equal to 0.552. As well, we demonstrated an association between elevated miR-155 expression levels and poor response to treatment with increased cases of relapse, partial remission or resistance to treatment.

Conclusions: This study suggests that miR-155 has an expression profile that differs according to B-cell malignancies subtype. Besides, miR-155 expression levels may modulate the patient's response to treatment. These results support a role for miR-155 to provide helpful diagnostic/prognostic information in B-cell malignancies and may highlight novel pathways to be targeted for therapeutics in the future.

Keywords: Diffuse large B-cell lymphoma (DLBCL); Chronic lymphocytic leukemia (CLL); Follicular lymphoma (FL); Burkitt's lymphoma (BL); microRNA-155 (miR-155)

Introduction

B-cell non-Hodgkin's lymphomas (BCNHLs) or B-cell malignancies are lymph-proliferative B-cell disorders that represent a set of more than twenty-five different malignancies. They include diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), follicular lymphoma (FL), Burkitt's lymphoma (BL) and various less frequent entities [1]. Non-Hodgkin's lymphomas (NHLs) are tumors originating from lymphoid tissues, mainly of lymph nodes. They are

the most prevalent lymphoid malignancy and representing one of the most common causes of death from cancers globally [2,3]. However, almost 85% of NHLs are of B-cell origin and only 15% are derived from T cells [4].

The diagnosis and staging of BCNHL are built mainly on histopathology. This is supplemented by immunohistochemistry, immunophenotyping as well as cytogenetics [1]. However, different B-cell malignancies share mutual molecular pathways, which is why they are still treated similarly ("one-fits-all" strategy). This current strategy in treating BCNHLs leads to inappropriate treatment decisions and hence incompetent cure [5]. Thus, finding more reliable molecular biomarkers for further accurate classification of B-cell malignancies is

crucial to individualize therapy through enhancing subgroup-specific treatment.

microRNAs (miRs) are small non-coding RNAs involved in gene regulation. They are considered recent key players in the pathogenesis and progression of several types of cancers as well as in their response to treatment. The addition of a miRs profile to the diagnosis of lymphoma, especially for the differentiation between B-cell malignancies subtypes, could represent an important novel future tool as promise biomarker [6]. Furthermore, the evaluation of peripheral blood miRs expression levels could be beneficial as non-invasive method for rapid diagnosis or monitoring of minimal residual disease besides being relatively stable during sample handling [7-9]. miR-155 was shown to be an oncomiR whose expression in B cells alone triggers malignant transformation. It has an important role in regulating oncogenesis, particularly in their response or adaptation to the tumor micro-environment [10]. Its expression is deregulated in several types of lymphoma and mice that over express miR-155 in B-cells developed B-cell malignancies. As well, cessation of miR-155 expression resulted in rapid regression of these malignancies, indicating a role for this miR in the maintenance of the oncogenic state [11]. Accordingly, miR-155 had been elucidated in recent studies as a potential promising diagnostic, prognostic or predictive biomarker in B-cell malignancies [12]. However, conflicting results have been presented plus lacking of its in-vivo peripheral expression profile within different subgroups of BCNHLs in one study [5]. Thus, its precise role in B-cell malignancies is not fully explored yet.

The aim of the present study was to provide a deeper insight into the role of miR-155 in the pathogenesis and sub-typing of BCNHLs. Also, our goal was involving the association between miR-155 and therapeutic fate of these patients. This could help in describing its possible diagnostic and prognostic capabilities and to delineate its feasibility as future therapeutic target as well.

Subjects and Methods

Subjects

This case control study was carried out at the Clinical Pathology, Internal Medicine and Pediatrics Departments, Faculty of Medicine, Minia University, Minia, Egypt as well as at Oncology Medicine Department, Oncology Centre, Ministry of health, Minia, Egypt. A total of 53 patients (Group I) were enrolled in this study along with 15 apparently healthy volunteers as a control group (Group II). The patients were including 22 patients with DLBCL (Group Ia), 15 patients with CLL (Group Ib), 9 patients with FL (Group Ic) beside 7 patients with BL (Group Id). The enrolled control subjects were age and sex matched with patients. Within the control group, the number of males was 8 subjects and the number of females was 7 subjects. Their ages were ranged from 10 to 70 years old. Within patients group, the number of males was 34 subjects and the number of females was 19 subjects. Their ages were ranged from 7 to 79 years old. Additionally, the patients group was further separated according to their response to treatment (after completing their treatment regimen by 6 month) into patients with complete remission (CR), partial remission (PR), resistance or no response disease (RD) and progressive disease or relapse (PD). These responses were evaluated in accordance with the response criteria of Cheson (National Cancer Institute Working Group guidelines for response to treatment) [13,14]. RD refers to failure of

primary treatment to achieve partial or complete remission but with stationary disease condition. CR employed when there is absence of clinical and radiographic evidence of disease or disease-related symptoms plus normal laboratory results while PR was attributed to $\geq 50\%$ decrease in tumor mass. Relapse was defined as progressive disease during initial treatment, failure to achieve complete remission or partial remission after completion of initial therapy, or progressive disease within 5 months after complete remission. The number of patients for follow up -after 6 months of finishing treatment-was 32 out of 53 cases -without treatment- that we started by them. This decrease in the number of follow up cases was because some cases died before the follow up time frame, some were referred to another oncology centers then we lost their footprints and some had refused the re-withdrawal of blood samples for follow-up detection of miR-155 expression levels.

Clinical data and laboratory samples collection

Thorough history questionnaires were filled for all subjects plus full clinical examination especially for monitoring hepatosplenomegaly (HSM) and lymphadenopathy. Moreover, lymph node biopsy and bone marrow aspiration were performed to all patients for recognition of the type, stage and performance of lymphoma. All pathological specimens were validated by three experienced pathologists in accordance with WHO classifications [15]. Patients' complete immunophenotyping was done using flow cytometer. In addition, chest X-ray and pelvi-abdominal ultrasound were carried out for all patients to detect any extramedullary involvement. Performance status was evaluated based on the Eastern Cooperative Oncology Group scale [16]. Follow-up was conducted every 2-3 weeks for assessment of treatment responses. The lymphoma stage was assessed using the Ann Arbor system and the modified Ann Arbor staging system for nasal NKTCL [17] via computed tomography (CT) or positron emission tomography (PET)/CT scans. Peripheral blood samples were withdrawn before initial treatment to measure all required laboratory tests. After completing the sessions of chemotherapy by 6 months, we collected second blood samples to re-evaluate the expression levels of miR-155 in accordance with patient's response to treatment. Patients with incomplete clinical or pathological data were excluded from this study. In the control group, subjects with chronic infections and autoimmune diseases were ruled out.

Two ml of blood were withdrawn in a sterile K3EDTA tube for complete blood count (CBC) and miR-155 gene expression analysis in PBMCs. As well, four ml of blood were withdrawn in plain tube to separate serum for LDH, liver and renal function testes. Blood urea, serum creatinine and liver enzymes (ALT and AST) were analyzed using automated colorimetric method (Mindray BS-800) while CBC was evaluated by automated blood counter (Sysmex KX-21N). Sera of patients were analyzed for lactate dehydrogenase (LDH) levels using Vitros 350 dry chemistry (Ortho clinical diagnostics, Johnson-Johnson Company; USA). miR-155 gene expression was quantitatively determined by real-time PCR technique.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA (including small RNAs as well as miRNAs) was extracted from EDTA whole blood samples using miRNeasy Mini Kit (Qiagen)

according to manufacture instructions. Total RNA was further used in reverse transcription followed by quantitative real-time PCR (RT-qPCR).

Initially, total RNA was reverse transcribed to cDNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems Inc.; Foster City, CA, USA). RT-qPCR was performed using TaqMan® MicroRNA Assays Kit (Applied Biosystems Inc.; Foster City, CA, USA) on a DTlite Real-Time PCR System (DNA technology, Moscow, Russia). In brief, cDNA product from RT reaction was added to TaqMan Universal PCR Master Mix II and TaqMan Small RNA Assay then Nuclease-free water was added in a total volume of 20 µL. The reactions were amplified at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. We used miR-103 to serve as an endogenous control (reference gene) [18]. At the end of the RT-qPCR, the thermal denaturation protocol was run to determine the number of products that were present in the reaction. The relative amount of miR-155 to miR-103 was calculated using the threshold cycle (Ct) cycle method. The Ct was calculated using DTmaster software. The relative amount of each miR-155 to miR-103 was described using the following formula: $\Delta\Delta Ct = \Delta Ct (\text{target gene}) - \Delta Ct (\text{reference gene})$.

The miRNA-155 reverse transcription primers and real-time PCR primers were designed as follows: miRNA-155-RT, GTCGTATCCATGGCAGGGTCCGAGGTATTCGCCATGGATACG ACTCCATC, miRNA-155-QPCRE, TAATACTGCCGGGTAAT, miRNA-155-QPCRR, TGGCAGGGTCCGAGGT. Meanwhile, internal reference primers were also designed according to the following sequences: miRNA-103-RT, CCTGGTTCGATGTCCTAGAGTTCATAGCCC, miRNA-103-QPCRE, TCCTGGTTCGATGTCCTAGAGT and miRNA-103-QPCRR, GGTGACAGCAGCATTGTACAG.

Statistical analysis

Differences of miR expression levels were analyzed using fold change and p-value. Fold change of >1.0 or <-1.0 along with p-value ≤ 0.05 were considered significant. Statistical Package for Social Sciences (SPSS) program version 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of data using student t-test to compare results between groups as regards quantitative data. The quantitative data were presented as mean ± standard deviation (SD) while the qualitative variables were described as number and percentage. Results were expressed as Tables and Figures. Graphics were done by Excel Microsoft Office 2010. A p-value of ≤ 0.05 was considered to be statistically significant, and all p-values correspond to two-sided significance tests. Correlation was performed by using Pearson correlation coefficient of variation (r). ROC curve was used to evaluate the diagnostic performance of miR-155 in B-cell malignancies subgroups versus apparently healthy control group or versus each others.

Results

The basic demographic, laboratory and clinico-pathological characteristics of the included subjects

The age of the control subjects was matched with that of the patients as there was no significant difference between patients and controls regarding their ages (p=0.14) (Table 1). Most DLBCL patients (81.8%; 18 of 22) were adults (>18 years), whereas the BL patients were only children (<12 years). On the other hand, all patients within CLL and FL subgroups were adults (>21 years) (Table 2). There was male predominance in both patients and control groups along with subgroups of B-cell malignancies (Tables 1 and 2). The distribution of adults and children between groups plus their related miR-155 levels were shown in Table 3.

Regarding laboratory results, there was statistically significant increase in the levels of ALT, AST, LDH and TLC in B-cell malignancies patients group compared to control group. Contrary, there was statistically significant decrease in both platelet counts and Hb levels between patients and control groups. However, there were statistically non-significant differences between these groups as regarding urea and creatinine levels (Table 1). The comparison of laboratory findings between B-cell malignancies subgroups was shown in Table 2.

All subjects in patients group were having lymphadenopathy. The percentage of cases with hepatomegaly and splenomegaly within B-cell malignancies as a whole and within its sub-groups was shown in Tables 2 and 4. There was an increase in the number of patients with hepatomegaly and/or splenomegaly than patients without. As well, the histological characteristics of patients plus their response to treatment were summarized in Tables 2 and 4.

Group		B-cell malignancies (N=53)	Healthy controls (N=15)	P-values
Variables				
Age (years)	Mean ± SD	41.81 ± 20.98	32.73 ± 20.35	0.14
Sex (male/ female)	(%)	64%	53%	-----
ALT (U/L)	Mean ± SD	44.7 ± 28.59	26.7 ± 7.5	0.008*
AST (U/L)	Mean ± SD	41.35 ± 17.5	24.9 ± 8.0	0.003*
Urea (mg/dl)	Mean ± SD	32.2 ± 11.3	29.2 ± 3.7	0.2
Creatinine (mg/dl)	Mean ± SD	0.71 ± 0.42	0.88 ± 0.2	0.07
LDH (g/L)	Mean ± SD	673.68 ± 293.07	290 ± 39.2	<0.001**
Platelets (1 × 10 ³ /µL)	Mean ± SD	184.4 ± 31.8	259.6 ± 39.28	0.001**
TLC (1 × 10 ³ /µL)	Mean ± SD	10.6 ± 9.6	6.3 ± 1.3	0.009*
Hb (g/dl)	Mean ± SD	10.4 ± 1.2	13.6 ± 1.1	<0.001**

Note: N: Number; *p-value ≤ 0.05; **p-value ≤ 0.001; SD: Standard Deviation; ALT: Alanine Transaminase; AST: Aspartate Transaminase; LDH: Lactate Dehydrogenase; TLC: Total Leucocytes Count; Hb: Hemoglobin

Table 1: Comparison between patients and control groups regarding demographic and laboratory data.

Variables		Groups			
		DLBCL (N=22)	CLL (N=15)	FL (N=9)	BL (N=7)
Age (years)	Mean ± SD	42.32 ± 18.76	57.7 ± 16.2	38.78 ± 10.11	10.0 ± 1.50
Sex (%)	Male/female	14 (63.6%)	8 (53.3%)	6 (66.7%)	6 (85.7%)
ALT(U/L)	Mean ± SD	43.4 ± 22.0	40.9 ± 22.0	58.38 ± 36.39	40.4 ± 26.1
AST(U/L)	Mean ± SD	56.71 ± 30.29	38.1 ± 17.2	42.13 ± 19.44	34.8 ± 14.55
Urea (mg/dl)	Mean ± SD	32.17 ± 11.38	36.4 ± 12.4	27.23 ± 9.28	30.0 ± 18.06
Creatinine (mg/dl)	Mean ± SD	0.75 ± 0.32	0.8 ± 0.6	0.67 ± 0.37	0.59 ± 0.16
LDH (g/L)	Mean ± SD	899.4 ± 583.9	563.6 ± 204.5	510.3 ± 195.3	556.9 ± 88.2
Platelets (1 × 10 ³ /μL)	Mean ± SD	145.71 ± 103.48	233.9 ± 156.8	217.75 ± 103.46	261.3 ± 91.0
TLC (1 × 10 ³ /μL)	Mean ± SD	7.23 ± 2.71	17.2 ± 13.0	7.64 ± 3.94	5.47 ± 2.34
Hb (g/dl)	Mean ± SD	9.59 ± 2.08	10.3 ± 1.9	11.1 ± 2.34	10.44 ± 2.54
Hepatomegaly (%)	Yes	12 (54.5 %)	9 (60.0%)	6 (66.7%)	1 (14.3%)
	No	10 (45.5 %)	6 (40.0%)	3 (33.3%)	6 (85.7%)
Splenomegaly (%)	Yes	9 (40.9 %)	12 (80%)	6 (66.7%)	5 (71.4%)
	No	13 (59.1%)	3 (20%)	3 (33.3%)	2 (28.6%)
LN enlargement (%)	Yes	22 (100%)	15 (100%)	9 (100%)	7 (100%)
	No	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Follow up-6 months after treatment		Total number=32 patients			
Group		DLBCL (N=11)	CLL (N=9)	FL (N=6)	BL (N=6)
Response to Treatment N (%)	RD	0 (0%)	1 (11.1%)	0 (0%)	0 (0%)
	PR	2 (18.2 %)	1 (11.1%)	2 (33.3%)	1 (16.7%)
	CR	7 (63.6%)	6 (66.7%)	3 (50.0%)	4 (66.6%)
	Relapse	2 (18.2%)	1 (11.1%)	1 (16.7%)	1 (16.7%)

Note: N: Number; DLBCL: Diffuse Large b-cell Lymphoma; CLL: Chronic Lymphocytic Leukemia; FL, Follicular Lymphoma; BL: Burkitt's Lymphoma; SD: Standard Deviation; ALT: Alanine Transaminase; AST: Aspartate Transaminase; LDH: Lactate Dehydrogenase; TLC: Total Leucocytes Count; Hb: Hemoglobin; LN: Lymph Node; RD: Resistant Disease; PR: Partial Remission; CR: Complete Remission

Table 2: Comparison of demographic, clinico-pathological and laboratory data between patients' subtypes.

		Adults	Pediatrics	p-values
B-cell malignancies (N=53)	N (%)	42 (79.2%)	11 (20.8%)	
	miR-155 expression level (Mean ± SD)	211.89 ± 148.95	107.28 ± 121.73	0.05*
Healthy controls (N=15)	N (%)	10 (66.7%)	5 (33.3%)	
	miR-155 expression level (Mean ± SD)	10.63 ± 7.14	11.94 ± 8.51	0.78

Note: N: Number; SD: Standard Deviation; *p-value ≤ 0.05

Table 3: Age distribution and age related miR-155 expression levels between studied groups.

Variables		Descriptive statistics (N and %)
Hepatomegaly (N=53)	Yes	28 (52.8%)
	No	25 (47.2%)
Splenomegaly (N=53)	Yes	32 (60.4%)
	No	21 (39.6%)
LN enlargement (N=53)	Yes	53 (100%)
	No	0 (0%)
Histologic subtype (N=53)	DLBCL	22 (41.5%)
	CLL	15 (28.3%)
	FL	9 (17.0%)
	BL	7 (13.2%)
Response to Treatment (N=32)	RD	1 (3.1%)
	PR	6 (18.8%)
	CR	20 (62.5%)
	Relapse	5 (15.6%)

Note: N: Number; DLBCL: Diffuse Large b-cell Lymphoma; CLL: Chronic Lymphocytic Leukemia; FL: Follicular Lymphoma; BL: Burkitt's Lymphoma; LN: Lymph Node; RD: Resistant Disease; PR: Partial Remission; CR: Complete Remission

Table 4: Descriptive statistics of clinico-pathological features within B-cell malignancies group.

The expression profile of miR-155 within B-cell malignancies group and their miR-155 based subtypes classification

There was highly statistically significant increase in miR-155 expression levels in patients with B-cell malignancies compared to control group ($p \leq 0.001$) (Figure 1). Also, the expression level of miR-155 was different between B-cell malignancies subtypes. DLBCL patients were showing the highest increase (29.26 fold increase than controls) followed by CLL (15.33 fold change) then FL (6.64 fold change). All these fold changes were statistically significant in comparison to each other as well as to controls ($p \leq 0.05$) (Figures 2 and 3). On the other hand, miR-155 expression level in BL was statistically insignificant in comparison to controls (1.25 fold change and $p=0.6$) but highly statistically significant lower in comparison to other NHL subgroups ($p \leq 0.001$) (Figures 2 and 3). The fold changes were calculated as how many fold expression as much as those of controls via dividing the $\Delta\Delta Ct$ of patient's miR-155 expression by control ones.

The diagnostic efficacies of miR-155 for B-cell malignancies subtypes

To determine cut-off levels that balanced the false-positive and the false-negative rates with the best positive predictive value, the receiver operating curve (ROC) analysis was performed. ROC curve of miR-155 for discriminating patients with B-cell malignancies as a whole from controls was shown in Figure 4A.

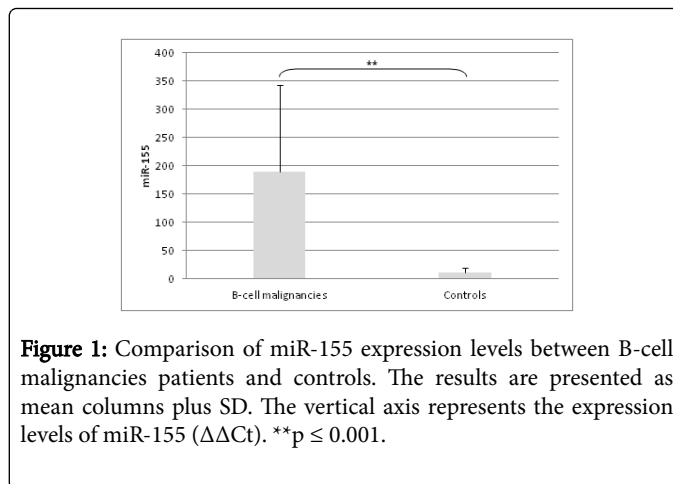


Figure 1: Comparison of miR-155 expression levels between B-cell malignancies patients and controls. The results are presented as mean columns plus SD. The vertical axis represents the expression levels of miR-155 ($\Delta\Delta Ct$). $**p \leq 0.001$.

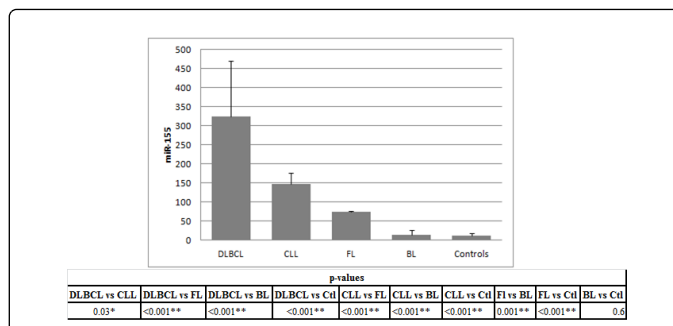


Figure 2: Comparison of miR-155 expression levels within B-cell malignancies subtypes. The results are presented as mean columns plus SD. The vertical axis represents the expression levels of miR-155 ($\Delta\Delta Ct$). DLBCL: Diffuse Large b-cell Lymphoma; CLL: Chronic Lymphocytic Leukemia; FL: Follicular Lymphoma; BL: Burkitt's Lymphoma. $**p \leq 0.001$.

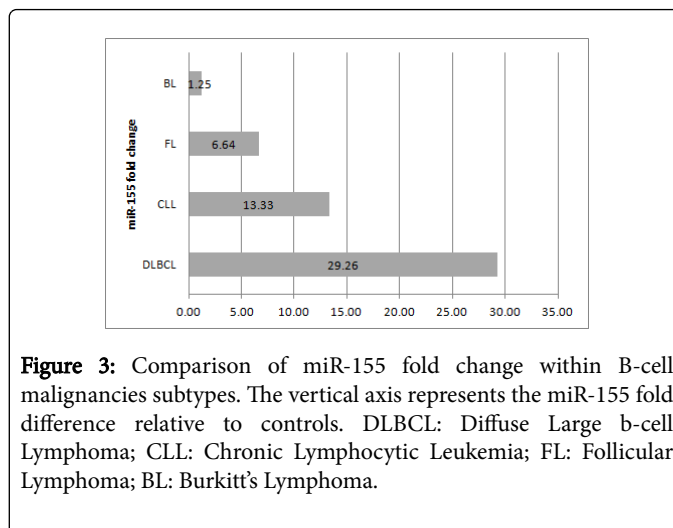
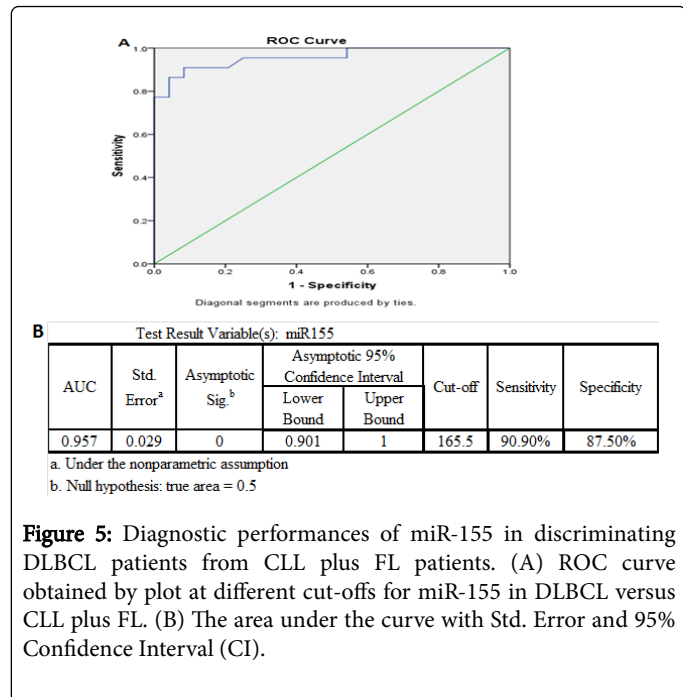
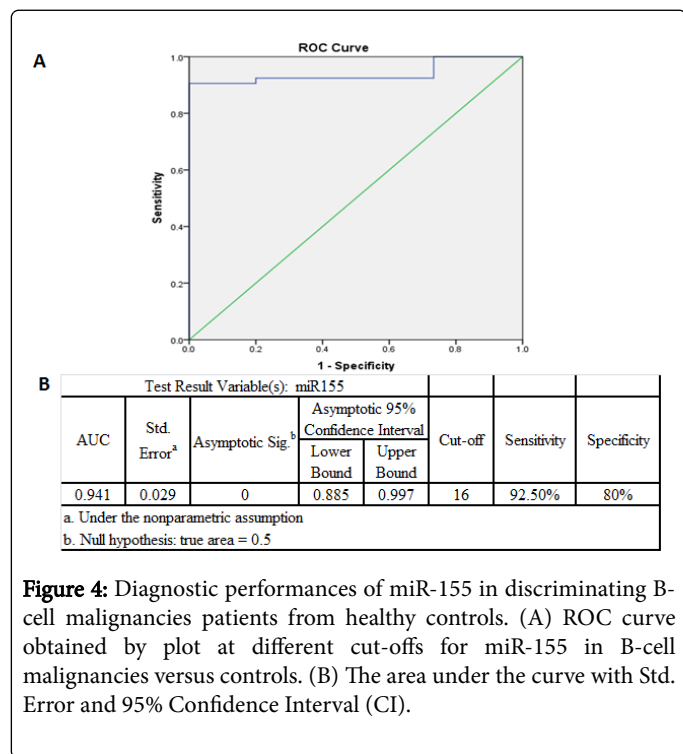


Figure 3: Comparison of miR-155 fold change within B-cell malignancies subtypes. The vertical axis represents the miR-155 fold difference relative to controls. DLBCL: Diffuse Large b-cell Lymphoma; CLL: Chronic Lymphocytic Leukemia; FL: Follicular Lymphoma; BL: Burkitt's Lymphoma.

The area under the curve (AUC) value was 0.941 [95% confidence interval (CI)=0.885–0.997, $p=0.000$] (Figure 4B). Moreover, the AUC value for discriminating patients with DLBCL from CLL plus FL was 0.957 [95% confidence interval (CI)=0.901–1.000, $p=0.000$] (Figure 5) while the AUC for discriminating CLL patients from FL ones was

1.000 (95% CI=1.000–1.000, p=0.000) (Figure 6). On the other hand, the AUC value for discriminating BL from controls was 0.552 (95% CI=0.264–0.841, p=0.698) (Figure 7). The different cut-off values plus different sensitivities and specificities were shown in Figures 4B, 5B, 6B and 7B.



Association between miR-155 expression levels and therapeutic fates

Our data show that the expression levels of miR-155 was different according to patient's response to treatment. The expression levels changed in a decreasing manner from relapse to PR, RD and CR. The highest levels were in cases with relapse and the lowest ones in CR cases (Figure 8). The mean of miR-155 expression levels were 115.26 for cases with CR response, 198.25 for cases with PR response, 341.14 for relapse response and 178.0 for resistant disease. Figure 8 shows mean ± SD of these values in association with different treatment responses.

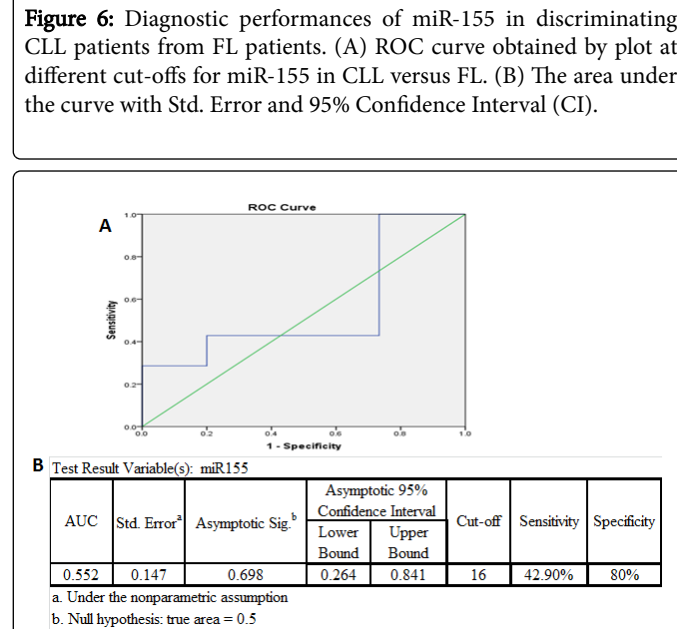
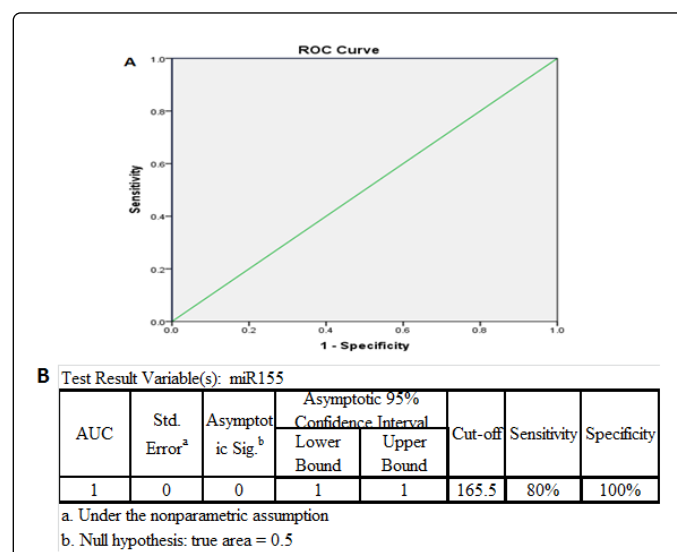


Figure 7: Diagnostic performances of miR-155 in discriminating BL patients from healthy controls. (A) ROC curve obtained by plot at different cut-offs for miR-155 in BL versus healthy controls. (B) The area under the curve with Std. Error and 95% Confidence Interval (CI).

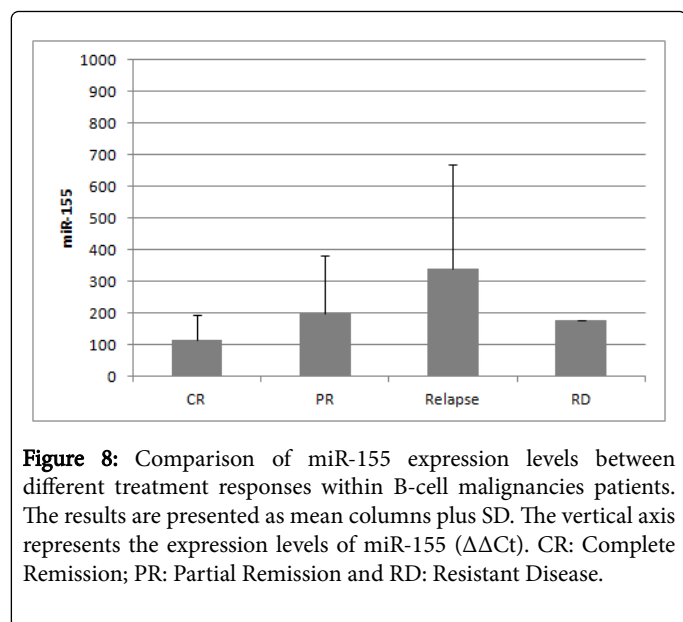


Figure 8: Comparison of miR-155 expression levels between different treatment responses within B-cell malignancies patients. The results are presented as mean columns plus SD. The vertical axis represents the expression levels of miR-155 ($\Delta\Delta C_t$). CR: Complete Remission; PR: Partial Remission and RD: Resistant Disease.

Correlation between patient’s miR-155 expression levels and their demographic, laboratory and clinico-pathological data

There was statistically significant fair positive correlation between miR-155 expression levels and age, AST and LDH levels ($r=0.279$ and $p=0.021$, $r=0.287$ and $p=0.018$, $r=0.411$ and $p<0.001$ respectively). Additionally, miR-155 expression levels were highly significantly correlated with hepatomegaly and also in a fair positive manner ($r=0.383$ and $p=0.001$). However, there was statistically significant fair negative correlation between miR-155 expression levels and Hb levels ($r=-0.316$ and $p=0.009$). The correlations between miR-155 expression levels and different patient’s data were shown in Table 5.

miR-155 and Variables	r	p-values
Age (years)	0.279	0.021*
Hepatomegaly (%)	0.383	0.001**
Splenomegaly (%)	0.026	0.833
ALT (U/L)	0.043	0.731
AST (U/L)	0.287	0.018*
Urea (mg/dl)	0.179	0.144
Creatinine (mg/dl)	-0.05	0.712
LDH (g/L)	0.411	<0.001**
Platelets ($1 \times 10^3/\mu L$)	0.154	0.21
TLC ($1 \times 10^3/\mu L$)	0.029	0.815
Hb (g/dl)	-0.32	0.009*

Note: $r=0.75-1$ (strong correlation); $r=0.5-0.74$ (moderate correlation); $r=0.25-0.49$ (fair correlation); $r=0.1-0.24$ (weak correlation); *p-value ≤ 0.05 ; **p-value ≤ 0.001 ; ALT: Alanine Transaminase; AST: Aspartate Transaminase; LDH: Lactate Dehydrogenase; TLC: Total Leucocytes Count; Hb: Hemoglobin

Table 5: Correlation of miR-155 with age and clinico-laboratory findings in patients group.

Discussion

B-cell malignancies are heterogeneous set of lymphoproliferative neoplasms. They are group of a broadly spread tumors worldwide with elevated mortality and morbidity [1]. The diagnosis and sub-typing of these malignancies depend mainly on histopathology of LN biopsies in addition to immunophenotyping and cytogenetics [5]. However, the invasive nature of biopsy plus the variability between pathologist’s experiences add a lot of limitations which make the sub-classification of these tumors harder. Accordingly, treatment of these malignancies is more or less alike regardless of the subtype. This “One fits all strategy” for treatment worsen the prognosis and adds up resistance to therapy [5]. Hence, finding more reliable and less invasive diagnostic and prognostic tools is crucial.

miRs are new promising diagnostic markers for many cancers. Researchers found either up or down regulations of miRs in different malignancies [19]. Additionally, miRs in peripheral blood are stable and their measuring is less invasive [20]. miR-155 was revealed recently to be involved in B-cells-related genes regulations either in physiologic or pathologic conditions. In particular, miR-155 gene expression was reported to be deregulated in different types of NHLs [21]. Though, the difference in the expression profile of this miR between B-cell malignancies subtypes is not fully explored yet. Identifying the expression profile of miR-155 within B-cell malignancies patients could be beneficial for identifying the molecular basis of each subclass of these lymphoid tumors and thus may be useful in individualizing their therapies hoping for better prognosis and prolonged survival. The aim of this study was to spot the variations in miR-155 expression levels among B-cell malignancies subtypes and to correlate between these levels and patient’s response to treatment as well.

In the current study, the incidence of B-cell malignancies was predominant in males (64%). This was in agreement with the study of Smith et al. in which most B-cell malignancies subtypes were significantly higher in males than females [22]. Also, most of the patients in this research, excluding BL subgroup, were above 40 years old with mean age of 41.8 years. Many previous studies had stated similar data concluding that the majority of B-cell malignancies occur mostly above the age of 50 [23-25]. On the other hand, BL is more common in children than in adults [26]. All the cases of BL in this study were children along with four children with DLBCL. We did a head to head comparison concerning the expression levels of miR-155 between children and adults within diseased or healthy controls. Our results revealed no statistically significant difference in miR-155 expression levels between pediatric and adult controls so this miR is well expressed early in life according to our data. As for B-cell malignancies group, there was a statistically significant difference in miR-155 expression levels between pediatrics and adults ($p=0.05$). This is because of the increased number of children within B-cell malignancies who suffering from BL than DLBCL subtype which contained the remaining children. As by our study, BL patients were expressing low levels of miR-155 relative to DLBCL. This also explains the elevated SD of this miR expression levels within pediatrics of B-cell malignancies (Mean \pm SD=107.28 \pm 121.73).

Further, our study has been shown that there were significant differences between LDH, ALT, AST and CBC parameters when malignant group was compared to apparently healthy controls. In addition, miR-155 expression levels were correlated positively and in significant manner with age, hepatomegaly, AST and LDH levels while associates negatively with Hb concentrations. Meanwhile, other than

these previous correlations no correlations were found between miR-155 expression levels and other laboratory and clinical data. These findings were consistent with the findings of Ferrajoli et al. study who revealed that miR-155 expression did not correlate with disease stage, absolute leukocyte count, or tumor burden before initiation of treatment [27]. These reported correlations in our data support the association between higher levels of miR-155 expression and bad prognosis as older ages, elevated LDH levels, low Hb levels as well as extra nodal sites involvement are considered risk factors for poor performances and inferior overall survival [28].

This study also showed that the expression level of miR-155 was up-regulated in B-cell malignancies as a whole group in comparison to control subjects. Moreover, our investigation revealed that miR-155 expression segregates between different pathologic subgroups of B-cell malignancies with highest expression in DLBCL. A number of previous studies were concerning about comparing miR-155 expression levels between different subtypes of B-cell malignancies. However, no previous studies were concerned about analysing in-vivo human peripheral blood circulating miR-155 expression levels within all these entities of B-cell malignancies that were studied in the current one (DLBCL, CLL, FL and BL) at ones and in parallel to each other's. We demonstrated different fold changes in miR-155 expression levels between studied groups when normalized to control subjects. DLBCL was expressing the significant greatest fold change of more than 29.0 times higher fold than miR-155 levels of control subjects followed by CLL (13.3) and FL (6.6). On the other hand, BL patients were showing statistically insignificant fold difference between their miR-155 expression levels and control group. Similarly, previous studies had elucidated an increased level of miR-155 expression in aggressive activated B-cell-like (ABC) subtype of DLBCL, primary mediastinal B-cell lymphoma (PMBL) and CLL. Their mean fold-change values had spanned from 3 to 19 in patients with DLBCL [29-37]. As well, a lot of studies have found down regulation of miR-155 expression levels within BL versus CLL, MCL and FL [38,39]. Contrary, the studies of Eis et al. [40] and Metzler [41] showed that miR-155 was up-regulated in pediatric burkitt's lymphoma which is opposite to what was shown in ours. However, these studies were performed on lymphoid cell lines and lymphoma tissue samples [40,41].

All together, these findings support the oncogenic nature of this miR in B-cell malignancies except for BL. This oncogenic scenery of miR-155 was also observed in the Carlo Croce's group transgenic mice that over express miR-155 in B cells and developed a polyclonal lympho-proliferative disorder followed by pre-B-leukemia/lymphoma at a young age then full-blown B-cell malignancy [42]. In Costinean study, miR-155 was up-regulated within in vitro ABC-DLBCL cell lines (OCI-Ly10, OCI-Ly3), but not in centroblasts (CBs) and germinal centre (GC)-DLBCL cell lines [42]. Additionally, the resistant of DLBCL to the growth-inhibitory effects of both TGF β 1 and bone morphogenetic protein (BMP) was attributed to miR-155 over expression through SMAD5-induced impaired cell cycle arrest as a result of p21 inhibition [43]. Several mechanisms were suggested to be involved in miR-155 induced lymphoma-genesis including inhibition of B-cell cycle arrest and induction of lympho-proliferation and migration. It was also reported recently that miR-155 down regulation promotes cell cycle arrest and apoptosis in DLBCL [44-46].

To the best of our knowledge, there were no reported studies that used ROC curve to tell about the clinical implication of miR-155 in distinguishing between different subclasses of B-cell malignancies. The current study employed ROC curve to tell between DLBCL and CLL

plus FL as well as to identify CLL from FL. The AUC was 0.957 and 1.00 respectively with high sensitivity and specificity for both. Our cut-off value was 165.5000. Fang et al. reported a cutoff value of 0.0022 and a sensitivity and specificity at 83% and 65%, respectively when DLBCL patients were versus healthy control [8]. Very recently, a study performed by Caivano and his group in 2016 carried out ROC analysis using serum miR-155 derived from extracellular vesicles (EV) [47]. This group found that the EV miR155 levels were significantly higher in CLL cases compared to controls. Conversely, they found no differences in this miR expression levels within both DLBCL and FL cases compared to controls [47]. The difference in the expression levels between our study and theirs considering DLBCL and FL may be referred to a possible difference in the genetic background of the involved subjects between their study and ours. Both of the studies did not include the genetic profile of the enrolled subjects and it was speculated before that only ABC molecular subtype of DLBCL express high levels of miR-155 not GC-DLBCL molecular subtype [42]. Also, the difference between both studies regarding the source of miR-155 could be in part responsible beside their extremely small sample size. Additionally, EV miR155 ROC curve analyses revealed significantly different pattern in CLL when compared to controls (AUC=0.874, p=0.004). Their sensitivity and specificity were 78% and 87.5% respectively at cut-off value of 17.0 [47]. However, their ROC comparison was regarding CLL alone and only versus controls unlike ours which was for different B-cell malignancies against each other's for the purpose of sub-classification which keeps the point of novelty to our research. As well, ROC curve was done here for BL cases against healthy controls. The AUC was 0.552 with reduced sensitivity and specificity. Thus, miR-155 is not efficient enough for identifying BL children according to our findings.

miRs have been drawn recently in the development of drug resistance because of their regulatory role in cell homeostasis [48]. Thus, monitoring their association with treatment response along with disease progression is fundamental. In this study, we found that disease remission as well as partial relapse and resistance to treatment are accompanied by elevated expression levels of miR-155. Additionally, the cases who died in this study were expressing very high levels of this miR. Values for this study are similar to those obtained by Hasselblom et al. and Uddin et al. groups [49,50]. Moreover, Babar et al. in 2012 revealed that nanoparticles of miR-155 antagonists were able to reduce tumors expressing high miR-155 levels in mice within a week [51]. Likewise, Ferrajoli et al. reported that the expression levels of miR-155 before therapy were significantly lower in CR patients than in poorer treatment responses. On top, they found that patients with high miR-155 levels before treatment had significantly shorter overall survival than patients with lower levels of miR-155 (p=0.012) [27].

On top, despite BL patients came up with very low levels or lack of miR-155 expression in most cases but there were three BL cases with exceptionally high miR-155 expression levels (data not shown). These patients were presented by an aggressive and advanced stage disease with multiple organs involvement. Their miR-155 expression levels were 33.0, 30.0 and 17.0. Also, another BL case with miR-155 expression level of 26.0 was died after the first cycle of therapy which was consistent with previous study performed by Iqbal et al. [38]. These findings mean that miR-155 does not act as tumor suppressor gene in BL because of the bad prognosis that accompanying BL cases who were expressing increased levels of miR-155. Other than, these data guide us to attribute the oncogenic nature of BL to unusual pathways different than those of other studied B-cell malignancies subtypes. This issue should be more deeply investigated in order to give

a clear and complete picture concerning the molecular bases that trigger malignant transformation in BL.

The current research has the benefit of being one of the fewest to compare between these specific B-cell malignancies subtypes at ones in the context of miR-155 expression levels as well as using the peripheral circulating miR-155. This adds more clinical significance to the study because of the less invasive lymphoma categorization and treatment monitoring way in comparison to other studies which used cell lines and/or LN biopsies. Also, our study was the first one to use ROC curve to distinguish between B-cell malignancies subtypes from each other's and determine the best cut-off values with the highest sensitivities and specificities for that purpose. Including children subjects in both patients and control groups also adds to the study besides being a follow up one. The limitations of this study were containing; 1) Limited size of the study subjects which was mainly because of financial issues. 2) Short follow up time and hence lack of survival calculation. 3) Relatively small number of pediatrics participants due to deficient available children who suffering from these malignancies within the time frame of the study. Larger prospective studies including bigger numbers of involved children along with longer follow-up periods should be considered. Measuring the miR-155 expression in tumor tissue plus blood samples will add to ongoing research. Furthermore, linking miR-155 gene expression to that of putative target genes would assist in exploring the molecular mechanisms that underlie the oncogenetic character of miR-155 within this group of malignancies. Finally, a more comprehensive study design might be required to correlate between miR-155 expression profile with Rai staging of B-cell malignancies, cytogenetics and immune-phenotyping as an attempt to develop B-cell malignancies predictive/diagnostic/classification less invasive panels independent on histopathology along with monitoring of therapy. This could give us deeper insights into these cancers which in turn will allow us to confidently enroll miR-155 as therapeutic target for these malignancies.

Conclusions

The present study showed that there is up-regulation in miR-155 expression levels in patients suffering from B-cells malignancies except for BL. Furthermore, there is statistically significant quantitative fold difference between these subtypes as well as different diagnostic performances suggesting that quantification of miR-155 could be used in the sub-classification of these malignancies in addition to its diagnostic potentials. This elevation in the circulating levels of miR-155 was correlated with poor response to treatment. Collectively, our data are telling that miR-155 could be useful as a novel biomarker for the categorization of B-cell malignancies subtypes plus its relevance as biomarkers for diagnosis and monitoring of treatment response. Thus, targeting this miR in the therapy of this group of malignancies should be considered in prospective studies.

Ethical Considerations

The study was performed in accordance with the World Medical Association's Declaration of Helsinki. It was approved by the research ethics committee of El-Minia University. The purpose and nature of the study were fully explained to the subjects and all of them signed written, informed consents before their enrollment in the study. All data were kept private and used for research purposes only.

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