Micro-RNA 155 in non-Hodgkin lymphoma

Hosny B. Hamed[a], Sahar A. Elgammal[b], Merna W. Narouz[a], Mohamed G. Elnaggar[a]

Background Micro-RNAs (miRNAs) are a class of small RNAs of 17–25 nucleotides in length. *miRNA-155* has a great role in lymphomagenesis through several mechanisms. Recent years have shown clearly a growth in knowledge of the etiology and pathogenesis of non-Hodgkin lymphomas (NHLs).

Aim This work aimed to study the expression of *miRNA-155* in patients with NHL and its correlation with clinical and pathological criteria of these patients; so that we could achieve a new biomarker in the prediction of progression of the disease.

Settings and study design This is a cross-sectional study with a nested control group. Cases and controls were recruited from the Clinical Pathology Department at South Egypt Cancer Institute.

Patients and methods This study was conducted on 32 patients with NHL. Peripheral blood and bone marrow samples were analyzed using 7500 Fast real-time PCR (Applied Biosystems).

Statistical analysis used Results were performed using IBM SPSS Statistics, version 20 (SPSS Inc.).

Results There was a significant correlation between expression levels of *miRNA-155* in patients with NHL and their clinical stages or international prognostic indices.

Conclusion It has been found that expression levels of *miRNA-155* in patients' samples were higher in cases of advanced clinical stage or higher international prognostic index. In addition, this study showed the correlation between *miRNA-155* and behavior of B-NHL subtypes, total leukocytic count, and hemoglobin levels of patients. These findings revealed the great role of *miRNA-155* in lymphomagenesis and also its usage as a new predictive biomarker in patients with NHL.

Egypt J Haematol 2021 46:48–57

Egyptian Journal of Haematology 2021 46:48–57

Keywords: biomarker, micro-RNAs, micro-RNA 155, non-Hodgkin lymphoma, real-time PCR

[a]Department of Oncological Clinical Pathology, South Egypt Cancer Institute, [b]Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt

Correspondence to Merna W.W. Narouz, MD, Department of Oncological Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assiut, 71511, Egypt. Mob: 01026004464;
e-mail: merna_wagih92@yahoo.com

Received: 12 January 2020 **Revised:** 18 February 2020
Accepted: 18 February 2020 **Published:** 29 October 2021

Introduction

Micro-RNAs (miRNAs) are a class of small (17–25 nucleotides), single-stranded noncoding RNAs that control gene expression in plants, animals, and unicellular eukaryotes. They have emerged as key post-transcriptional regulators [1–3]. MiRNAs act as post-transcriptional (negative) regulators through binding to complementary sequences located mainly within the untranslated regions of targeted genes. As a single miRNA can target several hundred genes, it is believed that 60% of all human genes are a potential target for miRNA regulation [4]. Moreover, a single target gene often contains binding sites for multiple miRNAs that can bind together [5], giving miRNAs the chance to form complex regulatory control networks. However, miRNAs have been shown to play key regulatory roles in many aspects of biology, including many physiological and pathological processes.

Recent years have shown clearly a growth in knowledge of the etiology and pathogenesis of non-Hodgkin lymphomas (NHLs). They are a heterogeneous group of neoplasms that include distinct categories, defined by clinical, morphological, immunological, and genetic characteristics [6]. NHLs are a diverse group (in character and content) of lymphoproliferative disorders originating in different cells, including B, T, or natural killer lymphocytes.

Micro-RNA 155 (*miRNA-155*) is a miRNA, which in humans is encoded by the *MIR155* host gene (*MIR155HG*) [7]. *MiRNA-155* has a role in various physiological and pathological processes. The *MIR155HG* was initially identified as a gene that was transcriptionally activated by promoter insertion at a common retroviral integration site in B-cell lymphomas called B-cell integration cluster [8].

Eis *et al.* [9] reported that *miRNA-155* gene is located at chromosome band 21q21.3, in the exon of a long noncoding RNA transcript from the B-cell integration cluster and encodes for the *miRNA-155*. It was later found that the *MIR155HG* was composed of

three exons that span a 13-kb region within human chromosome 21 (Hsa21 band q21.3) [10]. The *MIR155HG* is transcribed by RNA polymerase II, and the resulting ~1500 nucleotide RNA is capped and polyadenylated. The 23-nucleotide single-stranded *miRNA-155*, which is found in exon 3, is subsequently formed from the parent RNA molecule [9].

This miRNA has important roles in hematopoiesis, inflammation, immunity, and cancer [11–15] and is the archetypal multifunctional miRNA. Tili *et al.* [15] reported that in normal host, *miRNA-155* is upregulated in hematopoietic stem cells, myeloid progenitor cells, monocytes, granulocytes, macrophages, and dendritic cells during maturation and activation. Moreover, it is required for normal maturation and function of B and T lymphocytes. The *miRNA-155* signature is specific to bone-marrow-derived hematopoietic stem cells in humans [16]. In addition, the thymus exhibits the highest levels of *miRNA-155* expression compared with other human tissues [17]. *miRNA-155* is also expressed in $CD34^+$ cells of peripheral blood or bone marrow, and it is therefore revealed that *miRNA-155* may inhibit their differentiation into a more mature cell type [18]. Different mechanisms have been postulated for the *miRNA-155*-mediated pathogenesis of hematological malignancies. One of these proposed pathogenic mechanisms, especially for lymphomas such as diffuse large B-cell lymphoma (DLBCL), is the downregulation of B-cell lymphoma 6 protein and histone deacetylase 4 by *miRNA-155* [19]. The aim of this study was to evaluate the expression of *miRNA-155* in bone marrow and peripheral blood samples of patients with NHL by real-time PCR and its correlation with clinical and pathological criteria of these patients, so that we could achieve a new biomarker in the prediction of progression of the disease.

Patients and methods

This study was done on 32 patients of NHL: 28 newly diagnosed and four relapsed cases. These patients were presented to the South Egypt Cancer Institute Assiut University Hospital in the period between March 2018 and April 2019. Moreover, 30 apparently healthy participants, comprising 18 males and 12 females, aged 3–81 years, were included as a control group.

NHL was diagnosed based on the pathology of tissue biopsy and immunohistochemistry reports. The research program was approved by the ethical committee, Faculty of Medicine, Assiut University.

All participants were included after providing an informed consent. History was taken from all patients, and they were subjected to complete clinical examination, such as assessment of B-symptoms, lymphadenopathy, splenomegaly, hepatomegaly, and manifestations of anemia, thrombocytopenia if present. Some laboratory investigations were also carried out, including complete blood count, serum chemistry for lactate dehydrogenase (LDH), bone marrow aspiration and biopsy, and *miRNA-155* by real-time PCR.

Bone marrow and peripheral blood samples were collected in ethylenediaminetetraacetic acid vacutainer tubes.

miRNA-155 detection was done on real-time PCR using 7500 fast real-time PCR (Applied Biosystems, Foster City, California, USA). The blood sample was centrifuged at 2000 rpm for 5 min, and then the supernatant (plasma) was carefully transferred into an RNase-free tube for extraction of RNA. After centrifugation, samples were frozen at −20 to −80°C for long-term storage. Frozen plasma was incubated at 37°C in a water bath until completely thawed and salts were dissolved.

Purification of RNA was done as follows:

(1) In an RNase-free tube, 1000 µl of QIAzol lysis reagent was added to 200 µl of plasma and mixed by pipetting up and down.
(2) Chloroform (200 µl) was added to the tube containing the homogenate and closed securely, and then the tube was vortexed vigorously for 15 s. Centrifugation was done for 15 min at 15000 rpm at 4°C. After centrifugation, the sample was separated into three phases: an upper colorless aqueous phase containing RNA, a white interphase; and a lower red organic phase.
(3) The upper aqueous phase (600 µl of it) was transferred to a new collection tube, and then 900 µl of 100% ethanol was added and mixed thoroughly by pipetting up and down several times.
(4) The mixed sample (700 µl of it) was pipetted into an RNeasy Mini spin column in a 2-ml collection tube. Centrifugation was done at 10 000 rpm for 15 s at room temperature, and the flow-through was discarded. This step was repeated with the remainder of the sample, and the flow-through was discarded.
(5) Overall, 700 µl of RWT buffer was added to the RNeasy Mini spin column and centrifuged for 15 s at 10 000 rpm to wash the column, and then the flow-through was discarded.

(6) Then 500 µl of RPE buffer was added onto the RNeasy Mini spin column and centrifuged for 15 s at 10 000 rpm to wash the column, and then the flow-through was discarded. The previous step was repeated by using the same spin column with the same amount of RPE buffer.
(7) Following centrifugation, the RNeasy Mini spin column was removed from the collection tube carefully, so that the column does not contact the flow-through. Otherwise, carryover of ethanol would occur.
(8) The RNeasy Mini Spin column was placed into a new 2-ml collection tube, and the old collection tube was discarded with the flow-through. Then centrifugation was done at full speed for 2 min. The RNeasy Mini spin column was transferred to a new 1.5-ml collection tube, and 50-µl of RNase-free water was pipetted directly onto the RNeasy Mini spin column membrane and then centrifugation was done for 1 min at 10 000 rpm to elute the RNA.

Reverse transcription (RT) and quantification was done as follows:

(1) The RT Reaction Master Mix was prepared. In a polypropylene tube, the RT master mix was prepared by scaling certain volumes to the desired number of RT reactions.
(2) For each 15 µl of RT reaction, we added 0.15 µl of 100 mM dNTPs (with dTTP), 1 µL of MultiScribe Reverse Transcriptase (50 U/µl), 1.5 µl of (10× RT buffer), 0.19 µl of RNase inhibitor (20 U/µl), and finally 4.16 µl of nuclease-free water, so that the total volume would be 7 µl.
(3) Each 15-µl RT reaction consisted of 7 µl master mix (previously prepared), 3 µl primer, and 5 µl RNA sample.
(4) Contents were mixed gently and centrifuged to bring solution to the bottom of the tube.
(5) The tube was sealed, mixed gently and centrifuged to bring solution to the bottom of the tube. The tube was incubated on ice for 5 min and kept on ice until we were ready to load the thermal cycler. The thermal cycler was left in the 9600 emulation mode.

PCR amplification was done as follows:

(1) The recommended reaction volume was 20 µl.
(2) We prepared the plate so that each PCR reaction contained the following components: 1 µl of TaqMan miRNA assay (20×), 1.33 µl of the product from RT reaction (minimum 1 : 15 dilution), 10 µl of TaqMan 2× Universal PCR Master Mix, and finally 7.67 µl of nuclease-free water, so that the total volume would be 20 µl.
(3) We obtained a 1.5-ml microcentrifuge tube for each sample. The previous components were pipetted (prepared PCR reaction mix) either into the primer or RT product tubes.
(4) We capped the tube and inverted it several times to mix. The tube was centrifuged briefly.
(5) We transferred 20 µl of the complete PCR reaction mix (including assay and RT product) into wells. The plate was sealed with appropriate cover. We loaded the plate into the instrument.

Analysis of the data and result interpretation were done as follows:

(1) The cycle threshold (C_t) was determined and was defined as the fractional cycle number at which the fluorescence exceeded the given threshold. A widely used method to present relative expression of miRNA is the $2^{-\Delta\Delta ct}$ method (relative expression of miRNA presents the data of the miRNA of interest (C_t miRNA of interest) relative to internal control gene (RNU6B control miRNA), termed Δct [20–22].
(2) For each patient, we calculated Δct, which was the difference between ct of RNU6B gene and ct of miRNA-155 gene. For the control, calculated Δct was the difference between ct of the mean RNU6B gene of the control samples and ct of the mean miRNA-155 gene of the control samples; (this was the Δct of the mean control samples). For each patient, we calculated the ΔΔct, which was the difference between Δct of this patient and Δct of the mean control samples. Then, we applied the equation $2^{-\Delta\Delta ct}$ for each patient sample; if more than 1, it would be upregulated, whereas if less than 1, it would be downregulated. The data were presented as fold change. Fold change of the tested group is calculated relative to the control group (healthy adults) using the $2^{-\Delta\Delta ct}$ equation.

Different groups of patients with NHL were compared with healthy adults as a control group and tested for statistical significance using mean±SD and median (range) changes.

Results

Statistical analyses were performed using IBM SPSS Statistics version 20 (SPSS Inc., Chicago, Illinois, USA). Categorical data were presented as

frequencies and percentages, and c^2 tests were used for comparisons between groups. Continuous data were reported as mean±SD and median (range) and tested for normality using the Shapiro–Wilk test. Where continuous data were normally distributed, the Student's *t* test was used for comparisons between two groups, and where data were nonnormally distributed, the Mann–Whitney test was used. For more than two groups, Kruskal–Wallis test was used. Spearman correlation coefficients were used to assess the correlation between quantitative parameters. *P* value less than 0.05 was considered statistically significant.

This cross-sectional study was conducted to assess the *miRNA-155* in patients with NHL (*n*=32), and relative quantification (RQ) was obtained and interpreted compared with expression in healthy controls (*n*=30) (Table 1). There was no significant difference in age or sex between patients and controls.

The study involved 32 patients with NHL (21 males and 11 females): 40.6% DLBCL, 25% small lymphocytic/chronic lymphocytic lymphoma (CLL), 15.6% Burkitt's lymphoma (BL), 9.4% indolent B-cell splenic lymphoma, 3.1% of patients with mantle cell lymphoma (MCL), 3.1% with lymphoplasmacytic lymphoma, and 3.1% with follicular lymphoma (FL), who were aged from 3 to 80 years. There were 87.5% newly diagnosed and 12.5% relapsed patients. Overall, 59.4% of patients were diagnosed by excisional biopsy, 31.3% by tru-cut needle biopsy, and 9.4% of patients by fine-needle aspiration cytology. According to the clinical stage, 12.5% of patients were at stage I, 18.8% at stage II, 18.8% at stage III, and 50% of patients at stage IV. However, according to international prognostic index (IPI), 28.1% of patients were with low-risk IPI, 25% with low-intermediate-risk IPI, 15.6% with high-intermediate-risk IPI, and another 31.3% of patients with high-risk IPI. This study also included 30 controls (apparently healthy), comprising 18 males and 12 females, aged 3–81 years (Table 2). There were also different hematological parameters in the studied patients. As demonstrated in Table 2, 37.5% of patients had absolute lymphocytosis, abnormal lymphocytes, with shift to the left in their white blood cell differential, and white blood cells were normal in morphology in 34.4% of patients, and 28.1% showed absolute lymphopenia.

The expression of *miRNA-155* was higher in bone marrow compared with peripheral blood (38.3±19.4 vs. 7.5±4.8). Moreover, it was upregulated in bone marrow

Table 1 Age and sex of studied groups

	Cases (*N*=32)	Control (*N*=30)	*P* value[a]
Age			
Mean±SD	43.6±23.4	42.4±23.2	0.822
Median (minimum–maximum)	52.0 (3–80)	51.0 (3–81)	
Sex [*n* (%)]			
Male	21 (65.6)	18 (60)	0.647
Female	11 (34.4)	12 (40)	

[a]Mann–Whitney and c^2 tests were used.

Table 2 Clinical characteristics and hematological parameters of the studied patients (*N*=32)

	Frequency (*n*)	%
Type of case		
New	28	87.5
Relapsed	4	12.5
Diagnosed by		
Excisional biopsy	19	59.4
TCNB	10	31.3
FNAC	3	9.4
Pathological diagnosis		
DLBCL	13	40.6
SLL/CLL	8	25.0
BL	5	15.6
Others[a]	6	18.8
Stage		
I	4	12.5
II	6	18.8
III	6	18.8
IV	16	50.0
IPI		
Low risk	9	28.1
Low-intermediate-risk	8	25.0
High-intermediate-risk	5	15.6
High risk	10	31.3
Differential white blood cell count		
Normal in morphology	11	34.4
Absolute lymphocytosis, abnormal lymphocytes and shift to the left	12	37.5
Absolute lymphopenia	9	28.1
	Mean±SD	Median (minimum–maximum)
Hemoglobin	11.1±2.3	11.2 (6.9–14.5)
Platelets	255.8 ±120.7	258.0 (31–540)
White blood cells	16.9±6.2	7.6 (0.45–151.3)
Lymphocytes in peripheral blood	33.6±20.1	30.5 (4–92)
Lymphocytes in bone marrow	30.4±24.9	19.5 (5–92)

[a]Others (indolent B-cell splenic, mantle cell, lymphoplasmacytic, and follicular B-cell lymphoma). BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; FNAC, fine-needle aspiration cytology; IPI, international prognostic index; SLL/CLL, small lymphocytic/chronic lymphocytic lymphoma; TCNB, tru-cut needle biopsy.

Table 3 Micro-RNA 155 expression in peripheral blood and bone marrow of the studied patients (*N*=32)

	Bone marrow	Peripheral blood	*P* value[a]
RQ			
Mean±SE	38.3±19.4	7.5±4.8	<0.001^
Median (minimum–maximum)	10.1 (1.1–629.3)	0.8 (0.07–152.3)	
Regulation [*n* (%)]			
Upregulated	32 (100)	14 (43.8)	<0.001^
Downregulated	0	18 (56.3)	

RQ, relative quantification. [a]Mann–Whitney and c^2 tests were used. ^Significant *P* value (<0.05).

Table 4 Comparison of bone marrow micro-RNA 155 expression levels (relative quantification) regarding different parameters of the studied patients (*N*=32)

	RQ[a]	*P* value[b]
Sex		
Male	9.4 (1.1–87.2)	0.736
Female	18.7 (1.6–629.3)	
Type of case		
New	10.1 (1.1–629.3)	0.864
Relapsed	10.1 (3.2–46.2)	
Diagnosed by		
Excisional biopsy	16.9 (1.1–629.3)	0.891
TCNB	9.6 (1.6–47.9)	
FNAC	6.9 (3.3–43.6)	
Pathological diagnosis		
SLL/CLL	7. 4 (1.6–629.3)	0.241
DLBCL	15.1 (2.8–87.2)	
BL	9.9 (3.1–18.7)	
Others	2.5 (1.1–9.4)	
Stage		
I	2.7 (1.1–7.1)	0.048^
II	5.1 (2.3–43.6)	
III	14.4 (1.6–49.1)	
IV	18.8 (238–629.3)	
Differential white blood cell count		
Normal in morphology	9.9 (2.8–18.7)	0.005^
Absolute lymphocytosis, abnormal lymphocytes and shift to the left	43.4 (12.2–52.5)	
Absolute lymphopenia	3.3 (2.7–11.1)	
Aggressive	20.2 (1.6–87.2)	
Very aggressive	9.9 (3.1–18.7)	
IPI		
Low risk	6.9 (1.1–49.1)	0.036^
Low-intermediate-risk	5.2 (1.6–29.3)	
High-intermediate-risk	18.7 (2.6–43.6)	
High risk	32.3 (9.6–629.3)	

BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; FNAC, fine-needle aspiration cytology; IPI, international prognostic index; RQ, relative quantification; SLL/CLL, small lymphocytic/chronic lymphocytic lymphoma; TCNB, tru-cut needle biopsy. [b]Mann–Whitney and Kruskal–Wallis tests were used. ^Significant *P* value (<0.05).

of all cases compared with 43.8% in peripheral blood (Table 3).

In our study, the levels of RQ expression in bone marrow were higher in the patients with advanced stages (III and IV) (*P*=0.048), with high-risk IPI (*P*=0.036), and with patients who had absolute lymphocytosis, abnormal lymphocytes, and shift to the left in their white blood cells differential (*P*=0.005), and these differences were statistically significant (*P*<0.05) (Table 4).

Moreover, the levels of RQ expression in peripheral blood were higher in the patients with advanced stages (III and IV) (*P*=0.003), with high-risk IPI (*P*=0.014), and with patients who had absolute lymphocytosis, abnormal lymphocytes, and shift to the left in their white blood cell differential (*P*=0.003), and these

Table 5 Comparison of peripheral blood micro-RNA 155 expression levels (relative quantification) regarding different parameters of the studied patients (*N*=32)

	RQ[a]	*P* value[b]
Sex		
Male	0.98 (0.2–27.0)	0.226
Female	0.76 (0.07–152.3)	
Type of case		
New	0.79 (0.07–152.3)	0.494
Relapsed	0.96 (0.2–1.68)	
Diagnosed by		
Excisional biopsy	0.76 (0.1–152.3)	0.106
TCNB	1.3 (0.45–18.9)	
FNAC	0.31 (0.09–0.63)	
Pathological diagnosis		
SLL/CLL	0.76 (0.09–152.3)	0.477
DLBCL	0.63 (0.07–18.8)	
BL	0.98 (0.45–4.7)	
Other	0.76 (0.74–1.63)	
Stage		
I	0.66 (0.07–0.76)	0.003^
II	0.47 (0.09–0.77)	
III	0.68 (0.49–3.4)	
IV	2.2 (0.2–152.3)	
Differential white blood cell count		
Normal in morphology	0.59 (0.31–0.77)	0.003^
Absolute lymphocytosis, abnormal lymphocytes, and shift to the left	3.2 (1.7–15.5)	
Absolute lymphopenia	0.75 (0.54–1.3)	
IPI		
Low risk	0.74 (0.07–3.4)	0.014^
Low-intermediate-risk	0.73 (0.32–2.1)	
High-intermediate-risk	0.76 (0.09–18.9)	
High risk	3.7 (0.52–152.3)	

BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; FNAC, fine-needle aspiration cytology; IPI, international prognostic index; RQ, relative quantification; SLL/CLL, small lymphocytic/chronic lymphocytic lymphoma; TCNB, tru-cut needle biopsy. [a]RQ value expressed in median (minimum–maximum). [b]Mann–Whitney and Kruskal–Wallis tests were used. ^Significant *P* value (<0.05).

differences were statistically significant ($P<0.05$) (Table 5).

There was a statistically significant positive correlation between age and stage of NHL and RQ expression levels in bone marrow ($r=0.401$, $P=0.023$; and $r=0.464$, $P=0.007$, respectively). However, hemoglobin was negatively correlated with the RQ expression levels in the bone marrow ($r=-0.421$, $P=0.016$) (Table 6). There was also a statistically significant positive correlation between stage of NHL, white blood cells level, and lymphocytes in bone marrow and RQ expression levels in peripheral blood ($r=0.651$, $P=0.001$; $r=0.397$, $P=0.024$; and $r=0.438$, $P=0.022$, respectively). However, hemoglobin was negatively correlated with the RQ expression levels in peripheral blood ($r=-0.403$, $P=0.012$) (Table 6).

Moreover, there was a statistically significant positive correlation between RQ expression levels in bone marrow and peripheral blood of studied patients ($r=0.563$, $P=0.001$) (Table 7).

Discussion

In our study, we compared the expression levels of *miRNA-155* in peripheral blood or bone marrow samples of patients with NHL with that of healthy controls by RQ method using real-time PCR. RQ of healthy controls was always calculated as one and RQ of patients with NHL was calculated to be greater (upregulated) or less (downregulated) than one.

We found that *miRNA-155* expression was upregulated in peripheral blood of 43.8% and downregulated in 56.3% of patients with NHL compared with healthy controls. However, it was upregulated in all bone marrow samples (100%) of patients with B-cell NHL, whether they were relapsed or newly diagnosed, infiltrated or not, and whatever the patient's subtype or clinical stage was, compared with the control group; therefore, it was upregulated in DLBL, BL, small lymphocytic lymphoma/CLL, indolent B-cell splenic lymphoma, MCL, lymphoplasmacytic lymphoma, and FL in our study. This indicated that regardless of subtype

Table 6 Correlation between bone marrow and peripheral blood micro-RNA 155 expression levels and different parameters of studied patients (*N*=32)

Bone marrow	Micro-RNA 155 expression levels (RQ)	
	Correlation coefficient (*r*)	*P* value[a]
Age	0.401	0.023^
Stage	0.464	0.007^
Hemoglobin	–0.421	0.016^
Platelets	–0.228	0.210
White blood cells	0.229	0.207
Lymphocytes in peripheral blood	0.335	0.061
Lymphocytes in bone marrow	0.313	0.081
Peripheral blood		
Age	0.145	0.430
Stage	0.651	0.001^
Hemoglobin	–0.403	0.022^
Platelets	–0.302	0.092
White blood cells	0.397	0.024^
Lymphocytes in peripheral blood	0.327	0.068
Lymphocytes in bone marrow	0.438	0.012^

RQ, relative quantification. [a]Spearman correlation test was used. ^Significant *P* value (<0.05).

Table 7 Correlation between bone marrow and peripheral blood micro-RNA 155 expression levels in the studied patients (*N*=32)

	RQ bone marrow	
	Correlation coefficient (*r*)	*P* value[a]
RQ peripheral blood	0.563	0.001^

RQ, relative quantification. *Spearman correlation test was used. ^Significant *P* value (<0.05).

of B-NHL, there was upregulation of *miRNA-155* expression levels compared with healthy controls.

Altogether, these findings supported the oncogenic property of the *miRNA-155* in B-cell malignances. This oncogenic theory of *miRNA-155* was also observed in the study by Carlo Croce, where transgenic mice that had high expression levels of *miRNA-155* in B cells developed a polyclonal lymph-proliferative disorder followed by pre-B-leukemia/lymphoma at a young age and then complete B-cell malignancy [23].

Several mechanisms were believed to be involved in *miRNA-155*-induced lymphomagenesis, including inhibition of B-cell cycle arrest and induction of lymphproliferation. In addition, it was reported recently that *miRNA-155* downregulation causes cell cycle arrest and apoptosis in DLBCL [24–26].

One of these mechanisms reported that *miRNA-155* overexpression contributes to tumorigenesis; this was possibly owing to the dysregulation of the expression of members of the phosphatidylinositol-3-kinase and protein kinase B (PI3K-AKT) pathway, the transforming growth factor-β (TGF-β) pathway, and others [13,27,28].

Normally, TGF-β1 causes activation of SMA and MAD-related protein 5 (SMAD5). However, in DLBCL cells, which often express high levels of *miRNA-155*, there was marked suppression of SMAD5 expression, resulting in defective p15/p21 transcriptional induction and limited inhibition of the cyclin-dependent kinase (CDK). This CDK-cyclin complex mediates phosphorylation of the inactive tumor suppressor RB. The resulting hyperphosphorylated RB makes tumor cells progress through the cell cycle regardless of prohibitive extracellular TGF-β signals causing G0/G1 arrest, but in mature B cells that lack miRNA-155, elevated SMAD5 expression sensitizes the cells to TGF-β1-mediated induction of p15/p21; therefore, the high expression level of these CDK inhibitors blocks the CDK-cyclin complex, and the hypophosphorylated (active) RB promotes cell cycle arrest. This scenario explains the defective germinal center B-cell development in *miRNA-155* null mice [29].

Moreover, there is another mechanism which explains that *miRNA-155* represses the Src-homology inositol 5-phosphatase (SHIP-1). SHIP-1 is a critical phosphatase that negatively downmodulates the AKT pathway. It exerts its action in normal B cells; therefore, persistent overexpression of *miRNA-155* in B cells is thought to unblock AKT activity, causing B-cell proliferation. In addition to these mechanisms,

Gironella *et al.* [30] referred this effect of *miRNA-155* to the blockade of caspase-3 activity and decreased tumor protein 53-induced nuclear protein 1 (TP53INP1), which is a nuclear protein that is capable of inducing cell cycle arrest and apoptosis via the activation of caspase-3. In concordance with our previous results, Roehle *et al.* [31] reported that *miRNA-155* was overexpressed in FL and DLBCL. In addition, Thai *et al.* [14] and Shepshelovich *et al.* [32] found that *miRNA-155* overexpression as a frequent finding in patients with DLBCL. Similarly, previous studies had elucidated an increased level of *miRNA-155* expression in aggressive activated B-cell-like subtype of DLBCL, primary mediastinal B-cell lymphoma, and CLL [33–39]. Moreover, the studies of Metzler *et al.* [40] and Eis *et al.* [9] showed that *miRNA-155* was upregulated in pediatric BL.

In contrary, many studies, such as Robertus *et al.* [42], Di lisio *et al.* [33], and Iqbal *et al.* [41] have found downregulation of *miRNA-155* expression levels within BL versus CLL, MCL, and FL. The discrepancy in *miRNA-155* expression in BL between studies may be owing to the small number of patients in our study, differences in the ages of patients with BL involved in these studies, or owing to that they included in their studies formalin-fixed, paraffin-embedded tissue.

Furthermore, our study showed that there were differences between malignant group and apparently healthy controls in LDH levels and complete blood count parameters, especially in hemoglobin concentrations. LDH levels were always elevated in patients. However, hemoglobin concentrations were always decreased. In addition, *miRNA-155* expression levels in bone marrow samples correlated positively in a statistically significant manner with age and clinical stage, whereas associated negatively with hemoglobin concentrations. Its expression in peripheral blood samples of patients correlated positively in a statistically significant manner with clinical stage, white blood cells levels, and number of lymphocytes in bone marrow, whereas associated negatively with hemoglobin concentrations.

In our study, there was no correlation between *miRNA-155* expression levels (whether in bone marrow or peripheral blood samples of patients with B-NHL) and sex, type of case (newly diagnosed or relapsed patient), method of diagnosis, or behavior of B-NHL subtypes. However, there was a statistically significant relation between *miRNA-155* relative expression levels (whether in bone marrow or peripheral blood samples of patients with B-NHL) and clinical stage of patient's disease, bone marrow infiltration, and IPI score of the patient. Moreover, there were higher relative expression levels of *miRNA-155* in patients with NHL (whether bone marrow or peripheral blood samples) with advanced stage, infiltrated bone marrow, high IPI score, and with patients who had absolute lymphocytosis, abnormal lymphocytes, and shift to left in their white blood cells differential, and these results were statistically significant.

Meanwhile, other than these previous correlations, no correlations were found between *miRNA-155* expression levels and other laboratory or clinical data.

Eis *et al.* [9], Kluiver *et al.* [34], and Malumbres *et al.* [43] stated that *miRNA-155* expression levels showed no association with sex, age, clinical stage, or elevated LDH levels. Moreover, Zhong *et al.* [39] reported a lack of association between *miRNA-155* expression levels and sex, age, or clinical stage. However, in their studies, they showed that higher levels of *miRNA-155* were associated with the presence of high IPI score, which agreed with our study.

The IPI, which was originally designed as a prognostic factor model for aggressive NHL, also appeared to be useful for predicting the outcome of patients with low-grade lymphoma. This index has been also used to identify patients at high risk of relapse, based on specific sites of involvement, including bone marrow, central nervous system, liver, testis, lung, and spleen [44].

The IPI ranges from 0 to 5 and is defined as the sum of the presence of the following five factors: age more than 60 years, performance status, Ann Arbor stage III or IV, extranodal involvement of more than one site, and abnormal LDH level. Index values of 0 and 1 are classified as low (L), 2 as low intermediate (L/M), 3 as high intermediate (H/M), and 4 together with 5 as high (H) [45]. It predicted 5-year survival of groups being 73, 51, 43, and 26%, respectively [46].

Zhong *et al.* [39] reported that low *miRNA-155* was associated with a longer 5-year progression-free survival in newly diagnosed DLBCL cases. They found that the expression levels of *miRNA-155* and IPI status were statistically significant independent indicators of prognosis [31]. On the contrary, Lawrie *et al.* [37] reported the absence of an association between the expression of *miRNA-155* and IPI.

Regarding white blood cell differential count, it was found that there was a statistically significant difference in the relation between RQ expression levels of *miRNA-155* (whether in bone marrow or peripheral blood samples) of patients with NHL and the presence of absolute lymphocytosis, abnormal lymphocytes, and shift to the left in their white blood cell differential in patients' complete blood counts. On the contrary, the study by Ferrajoli *et al.* [47] revealed that *miRNA-155* expression did not correlate with absolute leukocyte count.

However, the correlation between higher white blood cell levels of patients and the presence of absolute lymphocytosis, abnormal lymphocytes, and shift to the left in their white blood cell differential and higher RQ expression levels in peripheral blood of patients explained a lot. As upregulated *miRNA-155* levels in peripheral blood of some patients with NHL depend mainly on the number of lymphocytes and their morphological abnormality, we could utilize this point – higher white blood cell levels and presence of morphological abnormality – in the future by just recommending peripheral blood samples for measuring *miRNA-155* expression levels in patients as a noninvasive method in prediction of the disease instead of the invasive bone marrow samples.There was also a statistically significant positive correlation between RQ expression levels of *miRNA-155* in bone marrow and that in peripheral blood samples of studied patients, which would explain that the worse the patient's clinical situation was, the higher RQ expression levels of *miRNA-155* in both bone marrow and peripheral blood samples.

RQ expression levels of *miRNA-155* in peripheral blood samples were up-regulated in 43.8% of patients, and although this was not a small percentage, we still need further research studies involving a larger number of patients to decide whether we could depend mainly on *miRNA-155* expression levels in peripheral blood as a novel noninvasive biomarker or not when there is leukocytosis or morphological abnormality in patient's complete blood count differential.

In spite of that, we could not deny its strong positive correlation, which was statistically significant, with advanced clinical stage of the patient (according to Ann Arbor classification), bone marrow infiltration, high IPI score, and leukocytosis and its morphological differentiation.

These reported correlations in our data supported the association between higher levels of *miRNA-155* expression and bad prognosis, as older ages, elevated LDH levels, low hemoglobin levels as well as extra nodal sites involvement are considered risk factors for poor performances and inferior overall survival [48].

Conclusion

As for our results, up-regulated *miRNA-155* expression level (whether in bone marrow or peripheral blood samples) of patients with NHL correlated positively in significant manner with clinical stage, high IPI score, and bone marrow infiltration. However, it correlated negatively in a significant manner with hemoglobin levels.

We found that there was a positive correlation between RQ expression levels of *miRNA-155* in bone marrow and that of peripheral blood samples of patients with NHL.

Moreover, in our study, the higher the RQ levels of *miRNA-155*, the advanced the clinical stage and higher the IPI score. The higher RQ levels were also accompanied with bone marrow infiltration.

Thus, we could conclude that *miRNA-155* could be considered as a novel predictive important biomarker of B-NHLs and a good indicator of clinical state, progression of the disease, and overall survival of the patients.

Moreover, the positive relationship between up-regulated *miRNA-155* of peripheral blood samples and high white blood cell levels and their morphological abnormality was considered promising results to utilize; therefore, we could resort to a less invasive method in the prediction of the progression of the disease, obtaining a novel circulating predictive biomarker.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**:281–297.

2 Molnár A, Schwach F, Studholme DJ, Thuenemann EC, Baulcombe DC. miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. *Nature* 2007; **447**:1126–1129.

3 Zhao T, Li G, Mi S, Li S, Hannon GJ, Wang X-J, *et al.* A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. *Genes Dev* 2007; **21**:1190–1203.

4 Friedman JM, Jones PA. MicroRNAs: critical mediators of differentiation, development and disease. *Swiss Med Wkly* 2009; **139**:466.

5 Lewis BP, Shih I-H, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell* 2003; **115**:787–798.

6 Armitage JO, Coiffier B, Dalla-Favera R. *Non-Hodgkin lymphomas*. Lippincott Williams & Wilkins; 2009.

7 Faraoni I, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: a typical multifunctional microRNA. *Biochim Biophys Acta* 2009; **1792**:497–505.

8 Tam W, Ben-Yehuda D, Hayward WS. bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol Cell Biol* 1997; **17**:1490–1502.

9 Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, *et al.* Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci* 2005;**102**:3627–3632.

10 Tam W. Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA. *Gene* 2001; **274**:157–167.

11 O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci* 2007;**104**:1604–1609.

12 Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* 2007; **316**:608–611.

13 Sandhu SK, Volinia S, Costinean S, Galasso M, Neinast R, Santhanam R, *et al.* miR-155 targets histone deacetylase 4 (HDAC4) and impairs transcriptional activity of B-cell lymphoma 6 (BCL6) in the Eμ-miR-155 transgenic mouse model. *Proc Natl Acad Sci* 2012; **109**:20047–20052.

14 Thai T-H, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, *et al.* Regulation of the germinal center response by microRNA-155. *Science* 2007; **316**:604–608.

15 Tili E, Croce CM, Michaille J-J. miR-155: on the crosstalk between inflammation and cancer. *Int Rev Immunol* 2009; **28**:264–284.

16 Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, *et al.* A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007; **129**:1401–1414.

17 Martin MM, Lee EJ, Buckenberger JA, Schmittgen TD, Elton TS. MicroRNA-155 regulates human angiotensin II type 1 receptor expression in fibroblasts. *J Biol Chem* 2006; **281**:18277–18284.

18 Georgantas RW, Hildreth R, Morisot S, Alder J, Liu C-g, Heimfeld S, *et al.* CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. *Proc Natl Acad Sci* 2007;**104**:2750–2755.

19 Higgs G, Slack F. The multiple roles of microRNA-155 in oncogenesis. *J Clin Bioinform* 2013; **3**:17.

20 D'Alessandra Y, Carena MC, Spazzafumo L, Martinelli F, Bassetti B, Devanna P, *et al.* Diagnostic potential of plasmatic MicroRNA signatures in stable and unstable angina. *PLoS ONE* 2013; **8**:e80345.

21 Li J, Xu J, Cheng Y, Wang F, Song Y, Xiao J. Circulating micro RNA s as mirrors of acute coronary syndromes: MiRacle or quagMire? *J Cell Mol Med* 2013; **17**:1363–1370.

22 Li L-m, Cai W-b, Ye Q, Liu J-m, Li X, Liao X-x. Comparison of plasma microRNA-1 and cardiac troponin T in early diagnosis of patients with acute myocardial infarction. *World J Emerg Med* 2014; **5**:182.

23 Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, *et al.* Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in Eμ-miR155 transgenic mice. *Proc Natl Acad Sci* 2006; **103**:7024–7029.

24 Croce C. MicroRNAs and lymphomas. *Ann Oncol* 2008; **19**(Suppl 4): iv39–iv40.

25 Musilova K, Mraz M. MicroRNAs in B-cell lymphomas: how a complex biology gets more complex. *Leukemia* 2015; **29**:1004.

26 Zhu F-q, Zeng L, Tang N, Tang Y-p, Zhou B-p, Li F-f, *et al.* MicroRNA-155 downregulation promotes cell cycle arrest and apoptosis in diffuse large B-cell lymphoma. *Oncol Res Feat Preclin Clin Cancer Therap* 2016; **24**:415–427.

27 Cinegaglia NC, Andrade SCS, Tokar T, Pinheiro M, Severino FE, Oliveira RA, *et al.* Integrative transcriptome analysis identifies deregulated microRNA-transcription factor networks in lung adenocarcinoma. *Oncotarget* 2016; **7**:28920.

28 Huang X, Shen Y, Liu M, Bi C, Jiang C, Iqbal J, *et al.* Quantitative proteomics reveals that miR-155 regulates the PI3K-AKT pathway in diffuse large B-cell lymphoma. *Am J Pathol* 2012; **181**:26–33.

29 Neumeister P, Sill H. Novel face of microRNA-155. *Blood* 2014; **123**:5–7.

30 Gironella M, Seux M, Xie M-J, Cano C, Tomasini R, Gommeaux J, *et al.* Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci* 2007;**104**:16170–16175.

31 Roehle A, Hoefig KP, Repsilber D, Thorns C, Ziepert M, Wesche KO, *et al.* MicroRNA signatures characterize diffuse large B-cell lymphomas and follicular lymphomas. *Br J Haematol* 2008; **142**:732–744.

32 Shepshelovich D, Ram R, Uziel O, Kushnir M, Lithwick-Yanai G, Hoshen M, *et al.* MicroRNA signature is indicative of long term prognosis in diffuse large B-cell lymphoma. *Leuk Res* 2015; **39**:632–637.

33 Di Lisio L, Sánchez-Beato M, Gómez-López G, Rodríguez ME, Montes-Moreno S, Mollejo M, *et al.* MicroRNA signatures in B-cell lymphomas. *Blood Cancer J* 2012; **2**:e57–e57.

34 Kluiver J, Poppema S, de Jong D, Blokzijl T, Harms G, Jacobs S, *et al.* BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J Pathol* 2005; **207**:243–249.

35 Lawrie CH, Chi J, Taylor S, Tramonti D, Ballabio E, Palazzo S, *et al.* Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. *J Cell Mol Med* 2009; **13**:1248–1260.

36 Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, *et al.* Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol* 2008; **141**:672–675.

37 Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, *et al.* MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. *Int J Cancer* 2007; **121**:1156–1161.

38 Rai D, Karanti S, Jung I, Dahia PL, Aguiar RC. Coordinated expression of microRNA-155 and predicted target genes in diffuse large B-cell lymphoma. *Cancer Genet Cytogenet* 2008; **181**:8–15.

39 Zhong H, Xu L, Zhong J-H, Xiao F, Liu Q, Huang H-H, *et al.* Clinical and prognostic significance of miR-155 and miR-146a expression levels in formalin-fixed/paraffin-embedded tissue of patients with diffuse large B-cell lymphoma. *Exp Ther Med* 2012; **3**:763–770.

40 Metzler M, Wilda M, Busch K, Viehmann S, Borkhardt A. High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. *Genes Chromosomes Cancer* 2004; **39**:167–169.

41 Iqbal J, Shen Y, Huang X, Liu Y, Wake L, Liu C, *et al.* Global microRNA expression profiling uncovers molecular markers for classification and prognosis in aggressive B-cell lymphoma. *Blood* 2015; **125**:1137–1145.

42 Robertus JL, Kluiver J, Weggemans C, Harms G, Reijmers RM, Swart Y, *et al.* MiRNA profiling in B non-Hodgkin lymphoma: a MYC-related miRNA profile characterizes Burkitt lymphoma. *Br J Haematol* 2010; **149**:896–899.

43 Malumbres R, Sarosiek KA, Cubedo E, Ruiz JW, Jiang X, Gascoyne RD, *et al.* Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. *Blood* 2009; **113**:3754–3764.

44 Shipp M. International non-Hodgkin's lymphoma prognostic factors project. A predictive model for aggressive non-Hodgkin's lymphoma. *N Eng J Med* 1993; **329**:987–994.

45 Hermans J, Krol AD, van Groningen K, Kluin PM, Kluin-Nelemans JC, Kramer MH, *et al.* International Prognostic Index for aggressive non-Hodgkin's lymphoma is valid for all malignancy grades. *Blood* 1995; **86**:1460–1463.

46 Kawthalkar SM. *Essentials of haematology*: JP Medical Ltd; 2013.

47 Ferrajoli A, Shanafelt TD, Ivan C, Shimizu M, Rabe KG, Nouraee N, *et al.* Prognostic value of miR-155 in individuals with monoclonal B-cell lymphocytosis and patients with B chronic lymphocytic leukemia. *Blood* 2013; **122**:1891–1899.

48 Nicolaides C, Dimou S, Pavlidis N. Prognostic factors in aggressive non-Hodgkin's lymphomas. *Oncologist* 1998; **3**:189–197.