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ORIGINAL ARTICLE

Serum miR-26a-5p and miR-199b-5p as a biomarker to predict therapy for Diffuse Large B-cell lymphoma with Hepatitis C Infection

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ABSTRACT

Background: Diffuse large B-cell lymphoma (DLBCL) has heterogeneity in its behavior, and pathological and molecular identification. Hematopoiesis is regulated by microRNAs thus its aberrant expression predisposes to myeloid and lymphoid neoplasms the current research aimed to explore serum miR-26a-5p and miR-199b-5p in patients with DLBCL and to evaluate its correlation with clinicopathological features and treatment protocols of NHLs among Egyptian patients with Hepatitis C virus (HCV)Infection. **Methods:** We enrolled 60 participants; 30 patients with DLBCL and 30 healthy persons as the control group. all subjects were screened for the presence of HCV-RNA in both plasma and PBMCs. miR-26a-5p and miR-199b-5p levels were determined by RT-PCR.**Results:** Serum miR-26a-5p and miR-199b-5p level values were downregulated in DLBCL patients with HCV and OCI compared to non-HCV and controls. There were significantly higher values of both miR-26a-5p and miR-199b-5p levels in patients with complete response(n=15,50%) compared to partial response (n= 5,16.7%) and primary refractory (n=10,33.3%), p<0.001. Noteworthy, there were statistically significant negative correlations between miR-26a-5p, miR-199b-5p, LDH, and total bilirubin. Linear regression tests revealed that among examined associated variables, only LDH was the independent variable associated with miR-26a-5p and miR-199b-5p, P <0.001. **Conclusions:**Serum miR-26a-5p and miR-199b-5p levels were down regulated in DLBCL patients, especially with HCV infection. Thus, these epigenetic markers could provide a new era of precision medicine to better refine diagnosis, prognostication, and rational treatment **Keywords:** miR-26a-5p; miR-199b-5p; Diffuse large B-cell lymphoma; Occult hepatitis C; R-CHOP.

INTRODUCTION

A preponderance of evidence suggests that Diffuse large B-cell lymphoma (DLBCL) has heterogeneity in its behavior, pathological, and molecular identification [1,2]. According to previous research results, the prevalence of DLBCL varies, accounting for approximately 25-30 % of all NHL patients worldwide [3]. Incidence is varied by

ethnicity, in developing countries particularly Egypt, the prevalence of DLBCL increases and reaches about 49 % of NHL.[٤]

Interesting research has corroborated that the etiology and pathogenesis of DLBCL are complex. It is well established that HCV infection is linked to other diseases such as DLBCL [5]. Interesting findings illustrate the role of HCV in the susceptibility and progression of DLBCL through the enhancement of lymphatic proliferation in particular B-cell.[٦]

There is compelling evidence suggesting that there are different types of chronic HCV such as occult hepatitis C virus infection (OCI) [7]. Despite the growing evidence that the most accurate assessment of OCI is a liver biopsy, it is still an invasive approach associated with numerous hazards [8]. It has been well-recognized that OCI detection in PBMCs is an alternative diagnostic technique [9]. Previous studies detected the association between OCI and lymphoproliferative disorders through its role in lymphomagenesis.[١٠]

It has been well established that HCV-positive patients with DLBCL have unique signature results from dysregulated genetic and epigenetic expression. Omics studies have demonstrated that each stage of hematopoiesis is regulated by other pathways, including microRNAs which are responsible for the regulation of hematopoiesis differentiation and so their peculiar expression promotes the susceptibility of lymphoma subtypes.[١١]

Mounting evidence suggests that the outcome of therapy for HCV-positive patients with DLBCL is controversial [12]. Considering all the above, the current study aimed to evaluate the levels of serum miR-26a-5p and miR-199b-5p and its association with clinicopathological features and R-CHOP protocols for DLBCL patients with HCV.

METHODS

The current research enrolled 60 participants:30 patients with DLBCL and 30 healthy participants as a control group. The age and sex of the case and control were matched. The DLBCL diagnosis was according to the WHO including clinical manifestations, an adequate biopsy specimen, and immunophenotype studies. All patients were treated with 6 cycles of R-CHOP; rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone protocols and all selected patients were in the remission stage. The Ethics Committee of the Faculty of Medicine, Zagazig University approved the protocol of the study with (IRB no. 10213). Each member signs an agreement to participate in the study. Laboratory testing was done according to operating techniques in Zagazig University Hospital as shown in The flow chart of the study demonstrated in Figure 1. Laboratory testing was done according to operating techniques in Zagazig University Hospital as shown in figure 1.

miR-199b-5p and miR-26a-5p relative expression levels by real-time PCR

The qPCR was performed to examine the relative expression of miR-31-5p compared with U6 as a reference gene using the SYBR Green method. The following primers were used, The relative expression level was determined using the 2-ΔΔCT method.

Name (gene)	Sequences
miR-199b-5p	F 5'TCAGCCCCAGTGTTTAGAC 3' R 5'- CTGAGGCTCACTGAGACCT -3'
miR-26a-5p	F 5'- CGAGCCATTCAAGTAATCCAG -3' R 5'- GAACATGTCTGCGTATCTC-3'
U6	F- 5'- GCTTCGAGGCAGGTTACATG-3' R- 5'- GCCTTGTAGCACGACCTTA -3'

STATISTICAL ANALYSIS

In this work, we used the statistical program for the social sciences (SPSS) version 28 (, and $P < 0.05$ was judged significant. For descriptive characterization, we used t-tests and Mann-Whitney-U-tests. For descriptive characterization, frequencies were calculated using crosstabs followed by $\chi 2$-tests. Correlations between miR-199b-5p and miR-26a-5p and studied parameters were performed.

RESULTS

In total, we enrolled 80 participants in our study. After excluding 20 subjects that did not fulfill the inclusion criteria, we included in the current study 60 participants: 30 patients with DLBCL, whose age was 54.6±12.5 years, and 30 subjects as the control group whose age was 53.6±11.5 years, p= 0.8255. We adjust the age and sex between cases and the control group to avoid any conflict that influences our results .

To investigate the clinicopathological characteristics of patients with DLBCL, we stratified our patients into 3 groups according to HCV infection status [NON-HCV(n=10), HCV(n=12), and OCI HCV (n=8). Our results suggested that there were significant differences between studied groups regarding baseline hepatic status; we found HCV and OCI HCV groups had a higher prevalence of chronic hepatitis and cirrhosis compared to NON-HCV as expected. In the subgroup analysis, we also noted an apparent spread and progression of lymphoma established by lymphadenopathy and hepatosplenomegaly (HSM), in HCV and OCI HCV groups compared to NON-HCV, P <0.001.*

Interestingly, the subgroup analysis in our current study stratified by disease status revealed a complete response to therapy was higher in NON-HCV compared to others. While partial response and primary refractory were common in patients with HCV and OCI HCV, P <0.05*. As regards International Prognostic Index for DLBCL (IPI), IPI was significantly higher in HCV positive patients compared to others, P <0.001*. Concerning laboratory tests of liver function and DLBCL, we detected significant differences between both groups as regards LDH, prothrombin time, albumin, ALT, and total bilirubin p<0.001 (Table 1).

To investigate the miR-26a-5p and miR-199b-5p levels in studied groups, we performed an ANOVA test and presented our data in Figures 2a and 2b. The interesting result of the existing research is that miR-26a-5p values were downregulated in DLBCL patients with HCV (0.30±0.109) in comparison to OCI (0.44±0.072), non-HCV (0.67±0.130) and controls (0.88±0.128), figure 2a, P <0.001.*

Concerning miR-199b-5p levels, our results revealed that miR-199b-5p levels downregulated in DLBCL patients with HCV (0.35±0.109) in comparison to OCI (0.46±0.065), non-HCV (0.720±0.130) and controls (0.9±0.128), figure 2b, P <0.001.*

Mysteriously, our patients were treated with 6–8 cycles of CHOP (n=21.70%) and R-CHOP(n=9,30%) protocols. To study the correlation between disease status and circulatory levels of mir-26a-5p and mir-199b-5p, we applied the ANOVA test, and we detected significantly higher values of both miR-26a-5p and miR-199b-5p levels in patients with complete response(n=15,50%) compared to partial response (n= 5,16.7%) and primary refractory (n=10,33.3%), p<0.001 (Table3). Interestingly, patients who received the CHOP protocol had significantly higher values of both miR-26a-5p and miR-199b-5p levels compared to patients treated with R-CHOP, p<0.001 (Table 2).

To study the correlation between miR-26a-5p and miR-199b-5p and laboratory tests, we applied Pearson correlation as shown in table 3. Our results suggested that there were statistically significant negative correlations between miR-26a-5p and miR-199b-5p and LDH, prothrombin time (PT) as well as total bilirubin, P <0.001*, (Table 3). on the contrary, we detected significant positive correlations between miR-26a-5p and miR-199b-5p and serum albumin P <0.001*,

(Table 3).

Taken together, these data show an expressive correlation between these epigenetic markers and liver function tests. To further evaluate the independent associations of miR-26a-5p and miR-199b-5p, we performed a linear regression test and we found that, among correlated parameters, only LDH was the independent variable associated with miR-26a-5p and miR-199b-5p, P <0.001*, (Table 4).

These results suggest that miR-199b-5p and miR-26a-5p levels were associated with clinicopathological features of DLBCL subgroups and response to therapy. Thus, we tested the power of miR-26a-5p and miR-199b-5p in predicting of DLBCL by ROC curve

The AUC was [0.957 (95% CI = 0.914–1.000) and 0.939 (95% CI = 0.885–0.994), respectively] with sensitivity [93 %,80% and respectively] and specificity [83.3% and 81.4, respectively] and the cutoff values (0.77 and 0.75, respectively), (Fig. 4a), P <0.001*

For further assessment of the current analytical test, we applied ROC analysis. The power of miR-26a-5p and miR-199b-5p for differentiation between OCI HCV group from non-HCV group the power of miR-26a-5p and miR-199b-5p in predicting DLBCL by ROC curve. The AUC was[0.958 (95% CI = 0.889–1.000) and 0.973 (95% CI = 0.920–1.000) , respectively] with sensitivity [95 %, and 94.6 respectively] and specificity [99% and 89.6 respectively] and the cutoff values (0.52and 0.55, respectively), (Fig. 4b), P <0.001*

Additionally, the power of miR-26a-5p and miR-199b-5p for differentiation between the HCV group from OCI HCV group. The AUC was [0.891 (95% CI = 0.748–1.000) and 0.818 (95% CI = 0.627–1.000), respectively] with sensitivity [75 %,74.9% and respectively] and specificity [74.9% and 65.5respectively] and the cutoff values (0.36 and 0.41, respectively), (Fig. 4c), P <0.001*

Table 1: clinicopathological characteristics in patients with DLBCL

Parameters	**DLBCL N=30**			
	NON-HCV N=10	**HCV N=12**	**OCI HCV N=8**	**$\chi 2$ P value**
Sex [n (%)]	7(70%)	7(58.3%)	4(50%)	**$\chi 2$**=0.764
Male	3(30%)	5(41.7%)	4(50%)	0.683
Female				
B symptoms [n (%)]				
Present	4(13.3%)	5(16.7%)	3(10%)	**$\chi 2$**=0.035
Absent	6(20%)	7(23.3%)	5(16.7%)	0.983
Base line hepatic status				
Normal	10(33.3%)	1(3.3%)	2(6.6%)	**$\chi 2$**=20.4
Chronic hepatitis	0(0%)	5(16.7%)	4(13.3%)	<0.001*
Cirrhosis	0(0%)	6(20%)	2(6.6%)	
Child score				
A	8(26.7%)	4(13.3%)	3(10%)	**$\chi 2$**=5.80
B	2(6.6%)	6(20%)	4(13.3%)	0.215
C	0(0%)	2(6.6%)	1(3.3%)	
BM aspirate [N (%)]				
Infiltrated	3(10%)	8(26.7%)	4(13.3%)	**$\chi 2$**=0.764
Not infiltrated	7(23.3%)	4(13.3%)	4(13.3%)	0.683

Parameters	DLBCL N=30			
	NON-HCV N=10	HCV N=12	OCI HCV N=8	$\chi 2$ P value
BM trephine [N (%)]				
Infiltrated	4(13.3%)	5(16.7%)	3(10%)	$\chi 2$=0.035
Not infiltrated	6(20%)	7(23.3%)	5(16.7%)	0.983
Site of involvement [N (%)]				
Lymphadenopathy	3(10%)	4(13.3%)	1(3.3%)	$\chi 2$=10.9
HSM	5(16.7%)	7(23.3%)	1(3.3%)	<0.001*
Lymphadenopathy and HSM	2(6.6%)	1(3.3%)	6(20%)	
Disease status				
Complete response	9(30%)	4(13.3%)	2(6.6%)	$\chi 2$=10.35
Partial response	1(3.3%)	2(6.6%)	2(6.6%)	<0.005
Primary refractory	0(0%)	6(20%)	4(13.3%)	
Stage [N (%)]				
I	3(10%)	0(0%)	1(3.3%)	$\chi 2$=9.029
II	2(6.6%)	1(3.3%)	1(3.3%)	0.529
III	1(3.3%)	3(10%)	1(3.3%)	
IIIS	2(6.6%)	1(3.3%)	1(3.3%)	
IV	2(6.6%)	7(23.3%))	4(13.3%)	
Performance status (WHO				$\chi 2$=4.66
score)	1(3.3%)	1(3.3%)	1(3.3%)	0.324
1	6(20%)	4(13.3%)	1(3.3%)	
2	3(10%)	7(23.3%)	6(20%)	
3				
IPI				
Low risk	8(26.7%)	3(10%)	1(3.3%)	
Low intermediate risk	1(3.3%)	2(6.6%)	4(13.3%)	$\chi 2$=14.8
High intermediate risk	1(3.3%)	1(3.3%)	1(3.3%)	<0.05*
High risk	0(0%)	6(20%)	2(6.6%)	
Alpha fetoprotein(ng/ml)	8.1 ±3.2	8.9 ±3.8	9.1 ±4.2	0.965
LDH	330.4±89.5	502.4±95.5	434.4±131.5	<0.001*
Prothrombin time (PT)	11.8±0.63	14.3±1.9	12.9±1.831	<0.001*
Albumin (g/dl)	4.05±1.13	3.22±0.96	4.1±0.8	<0.05*
Total bilirubin (mg/dl)	1.02±0.11	1.6±0.82	1.15±0.41	<0.05*
ALT(IU/L)	18.5±4.08	47.6±16.57	30.2±17.1	<0.001*

DLBCL;diffuse Large B-cell lymphoma, HSM); hepatosplenomegaly, IPI; International Prognostic Index for Diffuse Large B-cell Lymphoma, ALT; alanine transaminase.**$\chi 2$;** A chi-square test was used for categorical variables, to compare different groups. The ANOVA-test was used for quantitative variables. *P < 0.05 when compared with control group.

Table 2: comparison of mir-26a-5p and mir-199b-5p levels according to disease status.

Parameters	miR-26a-5p	miR-199b-5p
Complete response(n=15,50%)	0.60±0.185	0.65±0.19
Partial response (n= 5,16.7%)	0.36±0.085	0.41±0.15
Primary refractory (n=10,33.3%)	0.35±0.06	0.40 ±0.17
P1 value	<0.001*	<0.001*
P2 value	<0.001*	<0.001*
P3 value	0.974	0.873

R-CHOP; rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone
P1: compare between complete response and partial response.
P2: compare between complete response and primary refractory.
p3: compare between partial response and primary refractory.

Table 3: Correlation of circulatory levels of miR-26a-5p and miR-199b-5p a withclinical and laboratory characteristics in DLBCL

Parameters	miR-26a-5p		miR-199b-5p	
	r	p	*r*	p
Alpha fetoprotein(ng/ml)	-0.121	0.318	-0.232	0.217
LDH	-0.743	<0.001*	-0.636	<0.001*
Prothrombin time (PT)	-0.364	<0.05*	-0.556	<0.001*
Albumin (g/dl)	0.543	<0.001*	0.096	0.621
Total bilirubin (mg/dl)	-0.557	<0.001*	0.387	<0.001*
ALT(IU/L)	-0.281	0.132	-0.178	0.346

Table 4: linear regression analyses to examine the main independent variables against the relative expression level of miR-26a-5p and miR-199b-5p in DLBCL group.

		Unstandardized Coefficients		Standardized Coefficients			95% C.I.	
		B	S.E.	Beta	T	P value	Lower Bound	Upper Bound
miR-26a-5p	Constant	0.095	0.056		1.702	<0.05*	-0.020	0.210
	LDH	-0.006	0.004	-0.023	-1.296	0.207	-0.015	0.003
	PT	-0.023	0.016	-0.399	-1.452	0.160	-0.056	0.010
	Albumin	0.995	0.021	0.979	47.020	<0.001*	0.952	1.039
	Total bilirubin	-0.013	0.019	-0.135	-0.649	0.522	-0.052	0.027
miR-199b-5p	Constant	0.929	0.213		4.364	<0.001*	0.492	0.929
	PT	-0.021	0.019	-0.222	-1.093	0.284	-0.059	0.018

		Unstandardized Coefficients		Standardized Coefficients			95% C.I.	
		B	S.E.	Beta	T	P value	Lower Bound	Upper Bound
	ALT	-0.004	0.002	-0.415	-2.049	0.050	-0.008	0.000
	LDH	0.995	0.021	0.979	47.020	<0.001 *	.952	1.039
	Total bilirubin	-0.003	0.002	-0.360	-1.779	0.087	-0.008	0.001

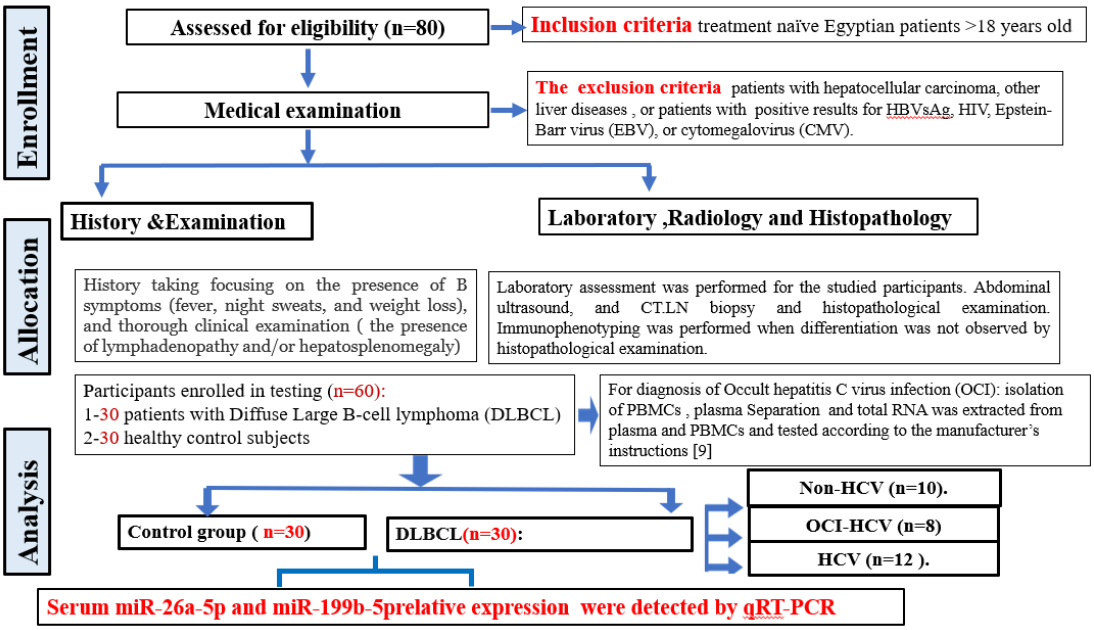

Figure 1: Flowchart of the study

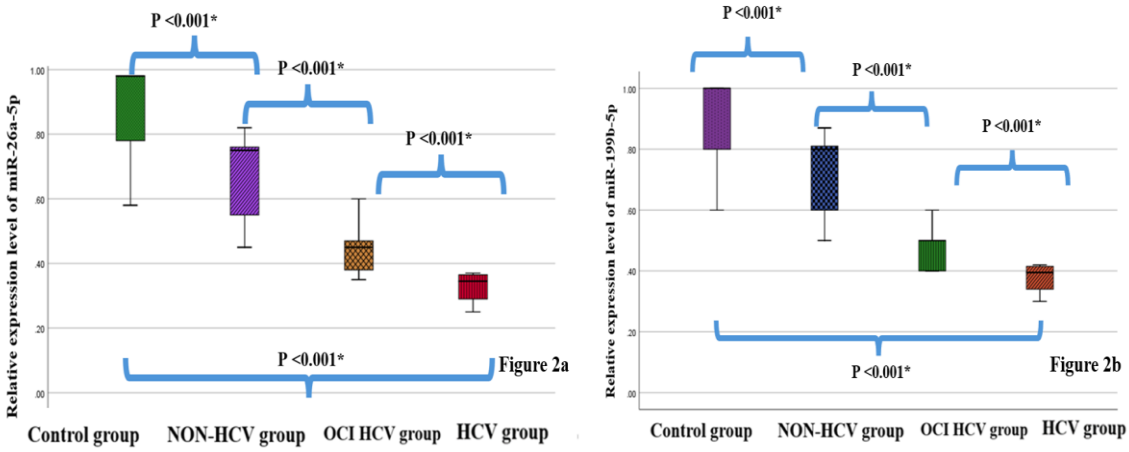

Figure 2a: comparison between studied groups regards miR-26a-5p relative expression levels.**Figure b** comparison between studied groups regards miR-199b-5p and

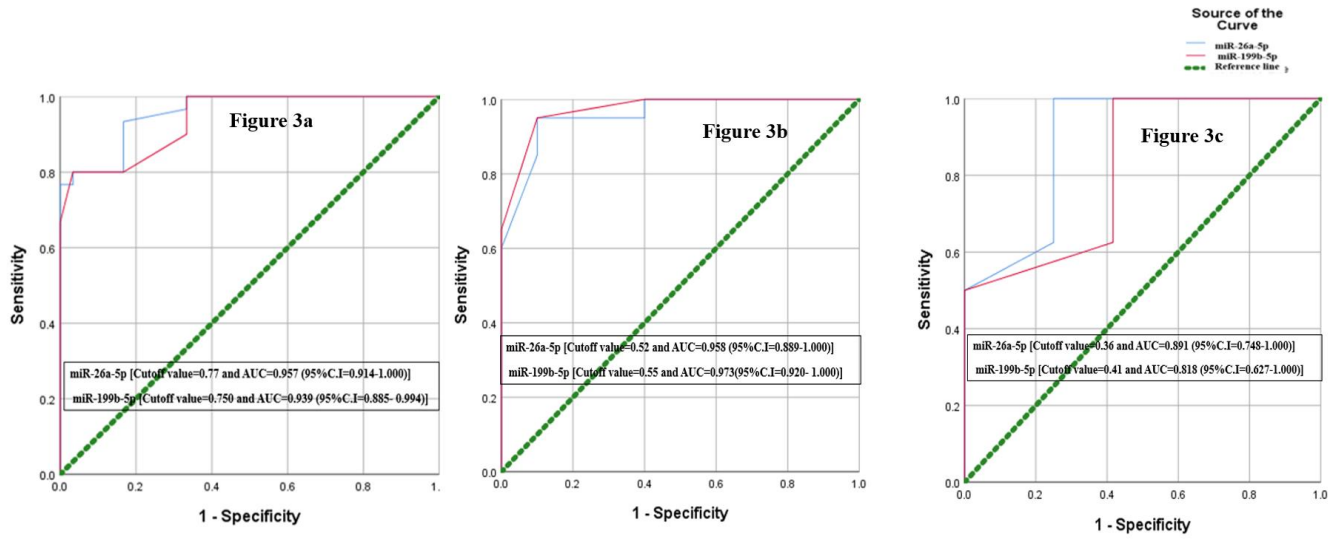

Figure 3a: ROC curve of miR-26a-5p andmiR-199b-5p in predicting of DLBCL.**Figure 3b:**ROC curve of miR-26a-5p and miR-199b-5p for differentiation between OCI HCV group from non-HCV group.**Figure 3c:**ROC curve of miR-26a-5p and miR-199b-5p for differentiation between non-HCV group from OCI HCV group.

DISCUSSION

Based on recent studies findings, HCV is not hepatotropic virus only, but also it is a lymphotropic virus that can infect and reproduce within peripheral blood mononuclear cells [13]. To test this hypothesis, we stratified our patients into 3 groups according to HCV infection statutes: NON-HCV, HCV, and OCI HCV. Our work demonstrates that patients with HCV and OCI HCV had a significantly higher prevalence of lymphadenopathy and HSM compared to NON-HCV.

Similar results were detected in Arcaini and his colleagues study, they observed more frequent spread and progression of lymphoma in HCV patients compared to NON-HCV patients [15]. This concept is also supported by Besson et al, who observed that HCV-positive DLCL were more commonly converted to severe lymphoma than DLCL in HCV-negative patients [16]. Also, Dlouhy et al found that HCV-positive DLBCL commonly influences other organs such as the spleen [17]. Our study quantified those patients with HCV and OCI HCV had significantly higher values of LDH, prothrombin time, albumin, ALT, and total bilirubin compared to NON-HCV. These findings were like the results of other interesting studies.[١٨-١٥]

Current study results revealed that the prevalence of complete response to therapy was higher in NON-HCV compared to other groups infected by HCV. While partial response and primary refractory were common in patients with HCV and OCI HCV compared to NON-HCV. Additionally, the frequency of IPI NHL was higher in HCV compared to others .

Our results are in harmony with Besson et al, they detected that HCV-positive DLCL had higher values of IPI, and this contributed to higher LDH levels [15]. In contrast, a study by Ennishi et al detected a similar response to R-CHOP in all studied patients.[١٩]

Emerging evidence demonstrated that miRNAs had variable roles in cancer as they lead to the progression of cancer and on the other hand they could inhibit cancer development and progression [20]. In this report, Bartolomé-Izquierdo et al detected

lower levels of circulatory has-miR-28 expression in NHL compared to the control [21]. Exciting research corroborated that dysregulated miRNA could contribute to B-cell maturation and lymphomagenesis dysregulation.[٢٢,٢٣]

The current research findings are pioneering as this study was the first Egyptian study that investigated serum miR-26a-5p and miR-199b-5p levels in DLBCL patients with or without HCV infection and we detected that both miR-26a-5p and miR-199b-5p values were decreased in DLBCL patients with HCV and OCI, in comparison to non-HCV and controls. Importantly, there were statistically significant negative correlations between miR-26a-5p, miR-199b-5p, LDH, and total bilirubin. Furthermore, LDH was the only studied parameter associated with both miR-26a-5p and miR-199b-5p in patients with DLBCL .

Current evidence indicates that proper therapy of DLBCL needs classification of DLBCL into subgroups according to histology, immunohistochemistry, and molecular and epigenetic characteristics as all these factors affect the response to treatment, prognosis, and survival of patients with DLBCL [24]. There is compelling evidence suggesting that some miRNAs such as miR-155, and miR-21 miRNA had oncogenic roles in B cell lymphomas, and others have tumor suppressor roles [25-27]. An interesting study detected lower miR-34a tissue expression in DLBCL in relation to the general population [٢٨,٢٩]

Our patients were treated with 6 cycles of R-CHOP protocols. The most important findings of our current research are that there were significantly higher values of both miR-26a-5p and miR-199b-5p levels in patients with complete response(n=15,50%) compared to partial response (n= 5,16.7%) and primary refractory (n=10,33.3%), p<0.001 .

Accumulating studies have reported that many miRNAs influenced the tumor sensitivity to specific drugs [30,31.] Likewise, Marques et al. suggested that overexpression of miR-34a can increase the sensitivity of DLBCL to doxorubicin [32]. In contrast, a study by Yuan et al detected that serum miR-125b and miR-130a are linked with R-CHOP resistance among patients with DLBCL .[٣٣]

Some authors suggest that some miRNAs can control the DLBCL response to R-CHOP [34,35]. Therefore, a combination of these miRNAs with the R-CHOP regimen might enhance the power of the therapy, thus improving the outcome. We in this study attempted to pierce out the diagnostic power of the power of miR-26a-5p and miR-199b-5p for diagnosis of DLBCL in addition to the power of these epigenetic markers in differentiation between HCV-positive DLBCL from non-HCV.

The current research revealed that the power of miR-26a-5p and miR-199b-5p in predicting DLBCL had sensitivity [93 %,80% and respectively] and specificity [83.3% and 81.4, respectively] at cutoff values (0.77 and 0.75, respectively. Remarkably the power of miR-26a-5p and miR-199b-5p for differentiation between the OCI HCV group from the non-HCV group had sensitivity [95 %, and 94.6 respectively] and specificity [99% and 89.6 respectively] at the cutoff values (0.52and 0.55, respectively. Furthermore, the power of miR-26a-5p and miR-199b-5p for differentiation between the HCV group from OCI HCV group. had sensitivity [75 %,74.9%, and respectively] and specificity [74.9% and 65.5respectively] at cutoff values (0.36 and 0.41, respectively). Finally, we observed that miR-26a-5p and

miR-199b-5p could be used as non-invasive markers for diagnosing DLBCL, predicting clinical outcomes, and responding to different protocols of therapy.

Declaration of interest:

The authors report no conflicts of interest. The authors along are responsible for the content and writing of the paper.

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The authors declare that this work has not taken any funding.

CONCLUSION

Serum miR-26a-5p and miR-199b-5p levels were downregulated in DLBCL patients, especially with HCV infection. The Discovery of new epigenetic markers not only improves our information a bout DLBCL pathogenetic mechanisms in lymphomas, but also provide prospects to improve the precision of diagnosis, prognostication, and rational treatment.

The study faced the limitation of a small sample size. Further studies enrolled large samples of patients treated by different protocols, are needed to confirm our research findings.

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