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Original Article

Clinicopathological significance of miR-101-3p and c-myc mRNA expression in diffused large B-cell lymphoma

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Abstract: MiR-101-3p has been suggested to be implicated in the pathogenesis of lymphoma, however little is known regarding clinicopathological significance of its expression in diffused large B-cell lymphoma (DLBCL). In contrast, although c-myc has been extensively studied in DLBCL, no direct evidence concerning clinicopathological significance has been well established, either. Given this, in our present study, to understand the significance of both miR-101-3p and c-myc expression on mRNA level in DLBCL, real-time quantitative PCR was used to detect the expression of miR-101-3p and c-myc on mRNA level in 100 cases of DLBCL samples. Association between expression of miR-101-3p and c-myc and clinicopathological variables available was statistically analyzed using Cross-Table test. It was shown that only significant association was observed between miR-101-3p expression and histopathological subtype and therapeutic regimen, no significant relationship was found with other clinicopathological variables. As for c-myc expression, only significant association was found with gender, IPI, and activity of LDH in serum; No significant relations were found with other clinicopathological variables. Together, our study presents the direct evidence regarding clinicopathological significance of miR-101-3p and c-myc expression in DLBCL, which warrants further confirmation in different cohorts with larger sample sizes.

Keywords: Diffused large B-cell lymphoma, miR-101-3p, c-myc, qPCR, prognosis

Introduction

Diffuse large B-cell lymphoma (DLBCL or DLBL) is the most common type of non-Hodgkin lymphoma among adults [1]. Although this cancer occurs primarily in the older, it can also occur in children and young adults [2]. DLBCL is an aggressive tumor which can arise virtually in any part of the body, and the first sign of this illness is typically the observation of a rapidly growing mass, sometimes referred to as B symptoms-fever, weight loss, and night sweats. The causes of DLBCL remains poorly understood, though several risk factors [3] have been identified to be linked to the DLBCL, including B symptom, Lactate dehyrogenase level in serum, International prognosis index (IPI), and so forth. DLBCL was marked by highly molecular and clinical heterogeneity [4], diagnosis of DLBCL has been challenging to young clinical pathologists and many patients don't respond well to the same common therapeutic regimen as we expected. Given this, new biomarkers or therapeutic targets may need, therefore, to be identified in order to improve the accuracy of DLBCL diagnosis and therapy.

One class of biomarkers that have been strongly suggested to be linked with the development of DLBCL is a subset of RNA molecules named microRNA (miR). miRs are small noncoding RNA (17-25 nucleotides in length) that bind mostly to target sequences within the 3' untranslated region of messenger RNA (mRNA), thus regulating the expression of thousands of mRNA including those with key roles in cancer pathogenesis [5]. In our previous work, to screen the differential miRs between DLBCL and normal lymph nodes, miR microarray was performed and several significantly differential

miRs were screened out (Supplementary Table 1). Among these, miR-101-3p was shown to be extremely significantly up-regulated in DLBCL relative to normal lymph nodes. Considering that miR-101, despite there were limited relevant reports in T-cell acute lymphoblastic leukemia (T-ALL) [6, 7], whose clinicopathological significance of its expression remains unknown in DLBCL, miR-101-3p was therefore picked up as miR of interest. Besides, although extensive relevant studies exhibited that c-myc has been found to play an important role in the development of DLBCL [8, 9]; no direct evidence regarding the clinicopathological significance of c-myc expression has been well established and understood in DLBCL, either.

Given this, to understand the clinicopathological significance of miR-101-3p and c-myc in DLBCL, real-time quantitative PCR (qPCR) was used to evaluate the expression of miR-101-3p and c-myc mRNA in 100 cases of DLBCL clinical samples. It showed that expression of miR-101-3p and c-myc was predominantly up-regulated in DLBCL. After further statistical analysis, there was significant association between miR-101-3p expression and therapeutic regimen and histopathological subtype; No significant relationship was found with other clinicopathological variables available, including overall prognosis, B symptom, International prognostic index (IPI), performance status, activity of Lactate Dehydrogenase (LDH) in serum, age and gender. Similarly, only significant association was observed with gender, IPI and activity of LDH in serum; No marked association was found with other clinicopathological variables available.

Materials and methods

Clinical samples

The study gets approved by the Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. The written informed content was obtained from each participant involved. A total of 100 cases of formalin fixed and paraffin embedded (FFPE) DLBCL clinical samples were collected and enrolled from the department of pathology, the First Affiliated Hospital of Xinjiang Medical University. The clinicopathological characteristics of the DLBCL samples we enrolled, including the age, gender, histopathological subtype, clinical stage, international prognostic index (IPI), activity of Lactate Dehydrogenase (LDH) in serum, Performance status, primary location, therapeutic regimen and overall prognosis were retrieved in the Hospital Information System (HIS) in the department of pathology. It should be noted that all clinicopathological variables were available with the exception of therapeutic regimen, only 33 cases whose corresponding information were available, the remainder was unavailable. All corresponding sections were confirmed and reviewed independently by two separate clinical pathologists (Wenli Cui and Xinxia Li). None of these patients underwent or received preoperative chemotherapy or radiotherapy.

Real-time quantitative PCR (qPCR)

Total RNA, including small RNAs and mRNA, was extracted using the Qiagen RNeasy FFPE Kit (Catalogue number: 73504, Qiagen, Ge-r man) according to the manufacturer's instruction. The concentration of RNA extracted was determined using a NanoDrop ND1000 spectrophotometer (Thermo Fisher, MA, USA). Reverse transcription of the mRNAs were performed using the PrimeScript^{RT} reagent kit (Sangon Biotech, Shanghai, China), and the expression levels of the mRNAs were determined using SYBR Premix Ex Taq (Sangon Biotech, Shanghai, China), β-actin was used as an internal loading control. Real-time PCR was performed on IQ5 real-time PCR instrument (CFX96, Bio-Rad, USA). The results of the qPCR analysis were determined based on the threshold cycle (Ct), and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, after normalization to the expression of β-actin. The stem loop primers of miR-101-3p, U6 primers, c-myc primers, and β-actin primers were designed and bought from Guangzhou RiboBio Co.LTD (Guangzhou, China). The detection of each sample was performed independently in three times with each time being triplicate. The mean value was the average of the three different times of detection. Considering that there was great deviation of mean of the expression of miR-101-3p and c-myc in DLBCL, the ratio of miR-101-3p to U6 equals to one or ratio of c-myc to β-actin equals to one was set as cut-off value. That whose relative expression value was more than

Table 1. Correlation between miR-101-3p expression and clinicopathological variables of DLBCL

Variables	Total (100 cases)	miR-101-3p expression		*P*
		High	low	
Age				
≥60	47	31	16	0.701
<60	53	33	20	
Gender				
Man	58	40	18	0.224
Female	42	24	18	
Histopathological subtype				
Germinal Center B cell	27	26	1	0.000
Non-Germinal Center B cell	73	38	35	
International prognosis index				
0-2	45	29	16	0.933
3-4	55	35	20	
Stage				
I-II	26	18	8	0.518
III-IV	74	46	28	
Lactate Dehydrogenase (U/L)				
≤240	40	24	16	0.496
>240	60	40	20	
Performance status				
0-2	71	44	27	0.509
3-5	29	20	9	
Primary site				
Intranodal	51	32	19	0.790
Extranodal	49	32	17	
B symptom				
Presence	27	19	8	0.420
Absence	73	45	28	
Therapeutic regimen				
R-CHOP	15	8	7	0.017
CHOP	10	8	2	
CHOPE	8	1	7	

one was taken to be high expression whereas that whose relative expression value was less than one was considered to be low expression.

Statistical analysis

Statistical analysis was performed using SPSS software 17.0 version. Continuous data were expressed as mean ± standard deviation of the mean (SDM); categorical data were expressed as percentages. Association between high and low expression of miR-101-3p and c-myc versus clinicopathological characteristics was carried out using the Chi-square test or Fisher's exact test when appropriate. The difference of survival between groups was analyzed using Log-Rank test in Kaplan-Meier survival curve. P<0.05 was considered to be statistically significant.

Results

The relationship between miR-101-3p expression and clinicopathological variables

To understand the clinicopathological significance of miR-101-3p expression in DLBCL, we've performed qPCR (Supplementary Figure 1). The expression of miR-101-3p was shown to be great deviation in DLBCL (data not shown). Of these 100 cases, in accordance with the cut-off value we set, the high expression of miR-101-3p was 64 cases, whereas the remainder 36 cases was low expression, therefore miR-101-3p can be judged to be predominant up-regulated in DLBCLin the absence of paired normal control tissues (**Table 1**). Based on the understanding of miR-101-3p expression, we've subsequently analyzed the relationship between miR-101-3p expression and clinicopathological variables using Chi-square test and Fisher's exact test when appropriate. It was shown that there was significant association between miR-101-3p expression and histopathological subtype and therapeutic regimen; while, no significant association was found with other clinicopathological variables available, including age, gender, IPI, clinical stage, activity of LDH in serum, performance status, primary site, B symptom, and overall prognosis (**Figure 1**). In consideration that miR-101-3p was markedly associated with therapeutic regimen, the prognosis data was further stratified by therapeutic regimen on which association between the prognosis and miR-101-3p expression was re-analyzed. There appeared to be a trend towards difference of prognosis despite no significant difference was observed (**Figure 1**). For patients received the cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) therapy, the prognosis of patients whose miR-101-3p was low tends to be better than those whose miR-101-3p was high.

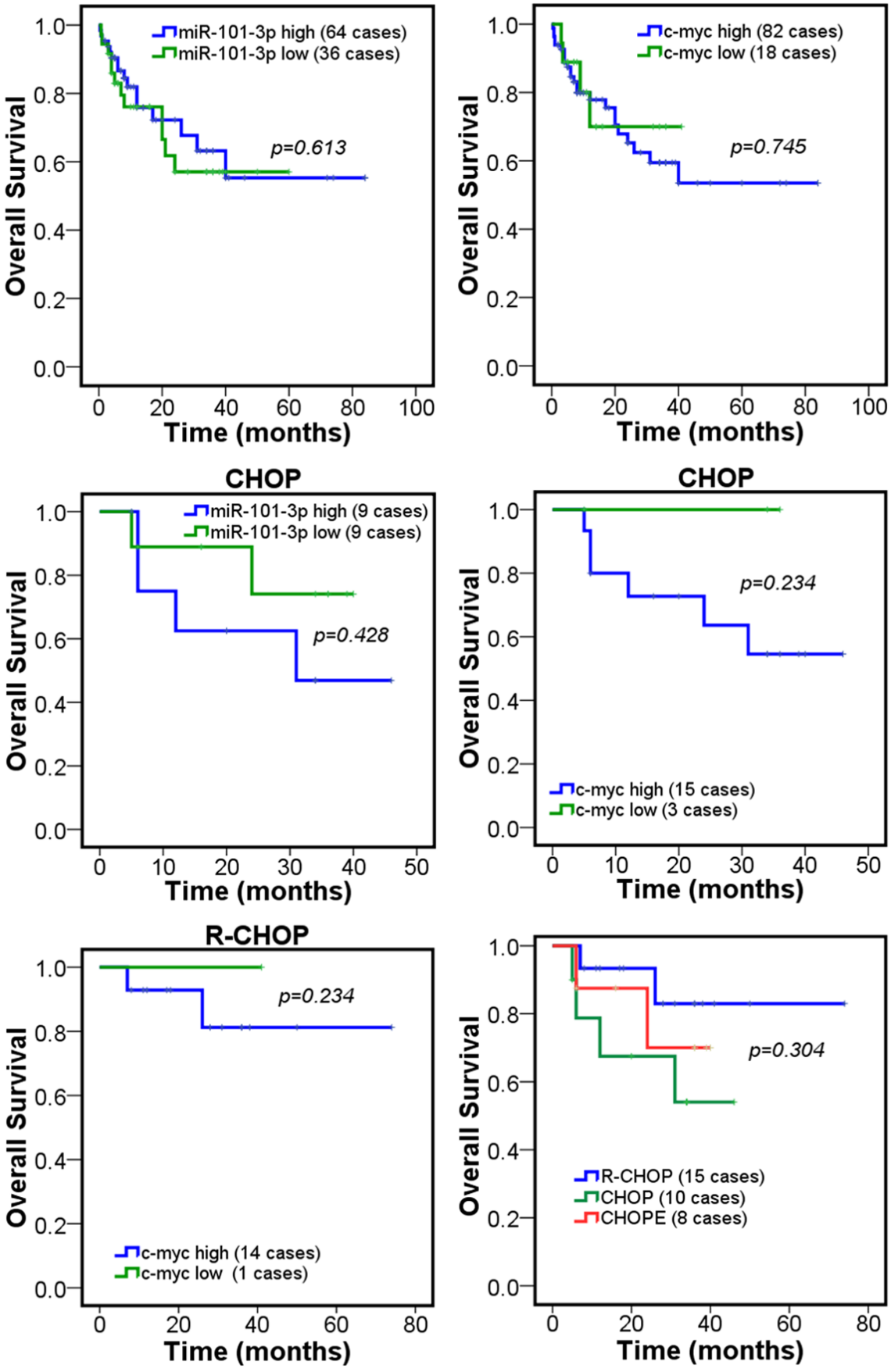
miR-101-3p high (64 cases)
miR-101-3p low (36 cases)
p=0.613
Overall Survival
Time (months)
c-myc high (82 cases)
c-myc low (18 cases)
p=0.745
CHOP
miR-101-3p high (9 cases)
miR-101-3p low (9 cases)
p=0.428
CHOP
p=0.234
c-myc high (15 cases)
c-myc low (3 cases)
R-CHOP
p=0.234
c-myc high (14 cases)
c-myc low (1 cases)
p=0.304
R-CHOP (15 cases)
CHOP (10 cases)
CHOPE (8 cases)

Figure 1. Prognostic associations of miR-101-3p and c-myc mRNA expression in DLBCL. A total of 100 cases of DLBCL tissues were enrolled whose overall prognosis information wasall available. The expression of both miR-101-3p and c-myc mRNA were actually heterogeneous, with the deviation of mean being great (data not shown here). Considering this, the relative expression value, whatever miR-101-3p or c-myc, equals to one was set as cut-off. That whose relative expression value was more than one was considered to be high expression, whereas that whose relative expression value was less than one was taken to be low expression. Log-Rank test was employed to analyze the statistical difference of overall prognosis between patients with high and low expression of miR-101-3p or c-myc.

Table 2. Correlation between c-myc mRNA expression and clinicopathological variables of DLBCL

Variables	Total (100 cases)	C-myc expression		*P*
		High	low	
Age				
≥60	47	40	7	0.446
<60	53	42	11	
Gender				
Male	58	53	5	0.004
Female	42	29	13	
Histopathological subtype				
Germinal Center B cell	27	19	8	0.066
Non-Germinal Center B cell	73	63	10	
International prognosis index				
0-2	45	33	12	0.041
3-4	55	49	6	
Clinical stage				
I-II	26	19	7	0.169
III-IV	74	63	11	
Lactate Dehydrogenase (U/L)				
≤240	40	28	12	0.011
>240	60	54	6	
Performance status				
0-2	71	56	15	0.203
3-4	29	26	3	
Primary site				
Intranodal	51	43	8	0.539
Extranodal	49	39	10	
B symptom				
Presence	27	25	2	0.094
Absence	73	57	16	
Therapeutic regimen				
R-CHOP	15	14	1	0.606
CHOP	10	8	2	
CHOPE	8	7	1	

The relationship between c-myc expression and clinicopathological variables

Likewise, to understand the clinicopathological significance of c-myc expression in DLBCL, The detection using qPCR was extended to c-myc (Supplementary Figure 2). C-myc expression was also displayed to be great deviation of mean, as miR-101-3p didin DLBCL (data not shown). Of these 100 cases, following the cut-off value we set, the high expression of c-myc was 82 cases, whereas the low expression was 18 cases. Given this, c-myc can also be diagnosed to be significantly up-regulated in DLBCL in spite of no corresponding normal controls (**Table 2**). On the basis of c-myc expression, next, we've analyzed the association between c-myc expression and clinicopathological variables using Chi-square test and Fisher's exact test where appropriate. It was found that there was significant association between c-myc expression and gender, IPI, and activity of LDH in serum; No significant association was observed with other clinicopathological variables available, including age, histopathological subtype, Clinical stage, Performance status, Primary site, B symptom, therapeutic regimen, and overall prognosis (**Figure 1**). Despite this, there was a trend towards significance of association between B symptom (p=0.094) and histopathological subtype (p=0.066) and c-myc expression. In the same way as miR-101-3p was analyzed, the prognosis data was further stratified by therapeutic regimen, as mentioned previously. Based on which, no significant association between c-myc expression and prognosis was observed but there seemed to be a trend towards difference of prognosis, that is, for patients received with CHOP or rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) therapy, the prognosis of those whose c-myc level was low tended to be superior to those whose c-myc level was high.

Discussion

This is the first report of miR-101-3p expression and clinicopathological significance in DLBCL. MiR-101-3p was shown to be up-regulated and to be markedly associated with histopatho-

logical subtype and therapeutic regimen in DLBCL. We also presented the direct evidence concerning the clinicopathological significance of c-myc expression using qPCR method in DLBCL, finding that c-myc expression was significant associated with the gender, IPI, and activity of LDH in serum. Both miR-101-3p and c-myc expression were shown to be independent of B symptom, clinical stage and overall prognosis, suggesting that both of the two markers were unlikely to be predictiveof the staging and prognosis in DLBCL.

In light of abnormal expression of miR has been linked to the development or early onset of DLBCL [10], to understand which potential miRs were associated with and involved in DLBCL, we've carried out the miR microarray analysis in our previous study. Among these significantly differential miRs we screened out from DLBCL, miR-101-3p was picked up in consideration of it has been hitherto unreported in the setting of DLBCL. Therefore, no direct evidence has been established regarding the expression and its clinicopathological significance of miR-101-3p in DLBCL. Considering this, to the best of our knowledge we've firstly reported and presented significance of miR-101-3p expression in DLBCL, finding that miR-101-3p was up-regulated predominantly on the whole in DLBCL despite in the absence of paired normal control tissues in our setting, which differs from those earlier relevant reports of miR-101 performed in T-cell acute lymphoblastic leukemia (T-ALL) [6, 7] where miR-101 was discovered to be remarkably down-regulated in the blood samples of patients with T-ALL. The discrepancy between our findings and these previous studies may be due to the fact that tissue-specificity of miR expression. In addition, miR-101-3p expression was shown to be markedly associated with histopathological subtype that classified into Germinal Center B cell (GCB) and Non-GCB type. It can be seen that in Non-GCG tissues, there was hardly any difference in terms of miR-101-3p expression. Nevertheless, the high expression of miR-101-3p was present in the vast majority of GCB tissues, indicating that miR-101-3p could be used as molecular biomarker telling the GCB from non-GCB subtype. Furthermore, we've tried to explore the association between miR-101-3p and therapeutic regimen whose corresponding information available was only 33 cases. These 33 cases were stratified by CHOP, R-CHOP and CHOPE followed by Fisher's exact test. It exhibited that miR-101-3p was pronouncedly decreased in patients received with CHOPE therapy compared with patients given with CHOP and R-CHOP, which is suggestive of miR-101-3p might be predictive of chemotherapeutic reaction for patients. Moreover, expression of miR-101-3p was shown to be independent of overall prognosis in DLBCL in our study, which was inconsistent with its prognostic significance in solid tumors as previously reported [11, 12].

C-myc, also aliased MYC, is one of the most frequently deregulated oncogenes in human malignancies. Deregulation of c-myc has been experimentally shown to be able to induce l-y mphomas, but requires cooperation with other lesions, including inactivation of the p53 pathway, structural alterations of BCL2 family members [13]. Thus, studies of its translocation [14] and re-arrangement [9] usually with BCL-2 gene [15, 16] have been substantially predominated in DLBCL. In comparison, clinicopathological significance of c-myc expression has been understudied and less reported. Extensive mechanistic studies have shown that c-myc contributed to the development of DLBCL; unfortunately, direct evidence regarding clinicopathological significance of its expression hasn't been well established. On the other hand, in view of the mechanistic regulation between c-myc and miR-101 [17, 18], which prompted us to select the c-myc as gene of interest at the outset in our setting. C-myc was displayed to be remarkably up-regulated in DLBCL although in the absence of corresponding paired normal control. Statistical analysis showed that c-myc expression was only markedly associated with gender, IPI, and activity of LDH in serum, which was partly different from the findings by Aref S et al [19] in non-Hodgkin's lymphoma that c-Myc over expression on protein level was not significantly related to the grade of IPI, the presence of B symptoms and histopathological type. But our findings were basically in line with observation made by Vettraino et al [20] that overactivation of c-myc induced increase in LDH expression in Burkitt lymphoma cells and by Shim H et al [21] that c-myc transactivated LDH. Unexpectedly, no relationship was found between c-myc mRNA expression and overall prognosis,

which was totally in disagreement with the one meta-analysis study regarding c-myc supporting that c-myc aberrations (on gene, protein, and mRNA level) was of prognostic value in DLBCL [22]. The reason leading to the inconsistency remains unknown that left to be further pondered over. In addition, there was a trend towards being significant of histopathological subtype and B symptom despite no significant relationship was factually observed. In our investigation, we also did not find any association between c-myc mRNA expression and therapeutic regimen.

There were some limitations in the study that deserves to be noted. Firstly, we failed to enroll the corresponding paired normal control in our analysis in consideration of the difficulty in collecting the normal lymph nodes. Interpretation of our results concerning miR-101-3p and c-myc expression in DLBCL therefore needs to be cautious in that, we did not mean that only miR-101-3p, picked up among the differential miRs we've screened using miR microarray, plays essential role in the pathogenesis of DLBCL; the remainder significantly differential miRs other than miR-101-3p may also play an important roles. Thus, further studies would be required. Secondly, our conclusion was based on the limited samples size [23], which was especially the case after being further stratified. Thus, interpretation of our results needs to be aware of it; thirdly, technique issues such as quality of RNA we extracted from FFPE tissues [24] and amplification of biomarkers we selected in our platform [25] may also potentially bias the final results. Another complementary detection approach to qPCR may be required meanwhile.

In conclusion, our study established the direct evidence concerning clinicopathological significance of miR-101-3p and c-myc mRNA expression in DLBCL, showing that both miR-101-3p and c-myc were up-regulated and miR-101-3p was presented to be markedly related with histopathological subtype and therapeutic regimen while c-myc expression was significant associated with the gender, IPI, and activity of LDH in serum. Our study warrants further confirmation in different cohorts with larger sample sizes.

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Disclosure of conflict of interest

None.

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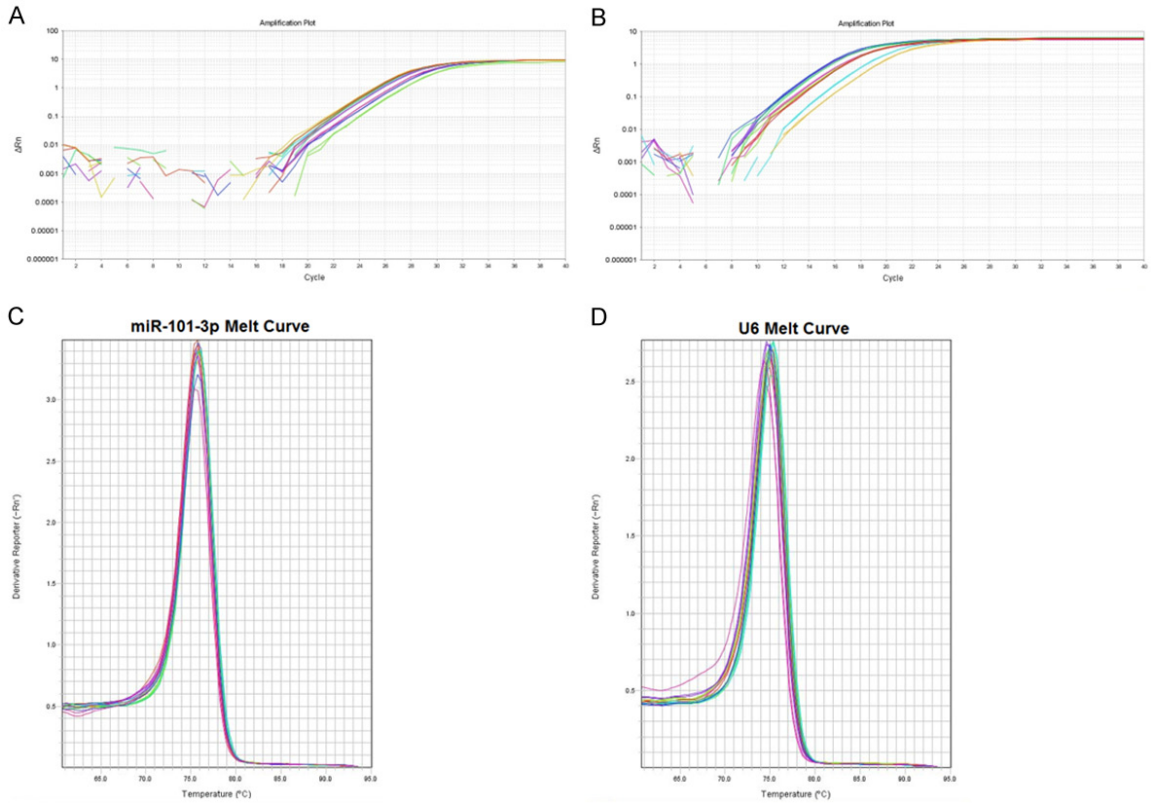
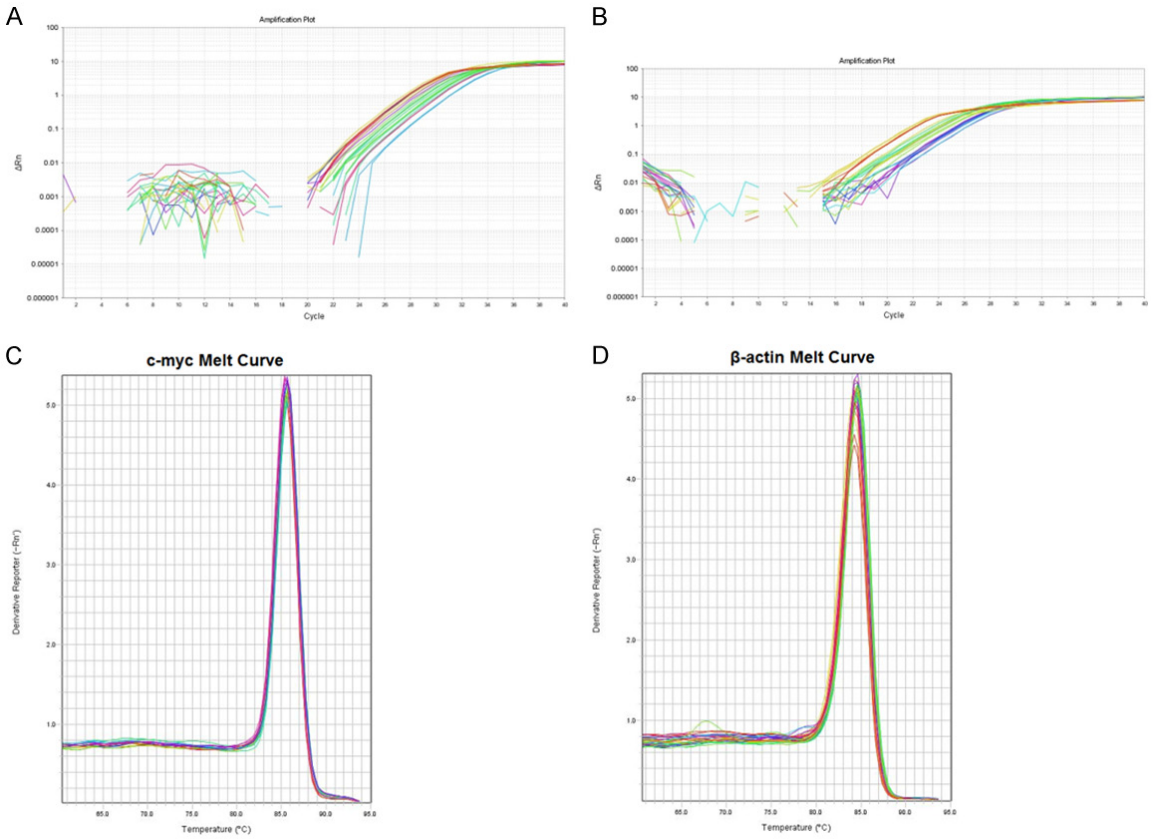
Supplementary Table 1. The significantly differential miRs screened out in DLBCL

MiRNAs	*P* value	Fold Change (absolute)	Regulation
Hsa-miR-101-3p	0.00000	70.12449	Up
Hsa-miR-20b-5p	0.03439	20.625223	Up
Hsa-miR-193a-3p	0.00085	52.908325	Up
Hsa-miR-28-5p	0.00121	48.691223	Up
Hsa-miR-142-5p	0.00511	39.288944	Up
Hsa-miR-19a-3p	0.01988	37.32824	Up
Hsa-miR-18a-5p	0.00879	34.147625	Up
Hsa-miR-1182	0.00059	36.81388	Down
Hsa-miR-518a-5p	0.00172	26.622723	Down
Hsa-miR-422a	0.00264	20.52196	Down



Supplementary Figure 1. Amplification curve and melting curve of miR-101-3p.



Supplementary Figure 2. Amplification curve and melting curve of c-myc.