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Original Article

Reproducibility of quantitative real-time PCR analysis in microRNA expression profiling and comparisons with microarray assays in diffuse large B-cell lymphoma patients

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Abstract: Diffuse large B-cell lymphoma (DLBCL), a malignant tumor, accounts for 30% to 40% of Non-Hodgkin lymphoma (NHL) patients. Dysregulation of microRNA (*miRNA*) expression has been documented in many tumors. This study aimed to assess miRNA expression in DLBCL to identify novel biomarkers that may improve diagnostic levels. The current study used miRNA microarray analysis to explore miRNA profiles in DLBCL patients, using formalin-fixed paraffin-embedded (FFPE) tissues of 3 samples from DLBCL patients and 3 from normal controls. This study also validated results using real-time quantitative PCR (RT-qPCR) in DLBCL (n=50), Burkitt's lymphoma (BL, n=40), and normal controls (n=25). Comprehensive analysis provided 12 miRNA expression profiles (*hsa-miR-181, hsa-miR-638, hsa-miR-328, hsa-miR-1268a, hsa-miR-1915-3p, hsa-miR-1268b, hsa-miR-1825, hsa-miR-4788, hsa-miR-4290, hsa-miR-3162-3p, hsa-miR-1202, hsa-miR-4649-3p*) of DLBCL showing overexpression from normal tissues ($P < 0.05$). After candidate miRNAs were selected, qRT-PCR further verified that 3 miRNAs (*hsa-miR-181, hsa-miR-683, hsa-miR1268*) were significantly overexpressed in DLBCL and BL tissues, compared to normal samples. Results indicate that aberrant miRNA expression was involved in the development and progression of DLBCL. Many of the miRNAs, identified in the current research, are new and have not been reported in previous studies, perhaps due to population differences. In conclusion, present results suggest that these miRNAs have potential use as diagnostic biomarkers, distinguishing DLBCL and BL patients from normal individuals and offering new targets for future therapies.

Keywords: MicroRNA, expression, diffuse large B-cell lymphoma, Burkitt's lymphoma

Introduction

Non-Hodgkin lymphoma (NHL) is the fifth most common cancer, worldwide, and comprises many different subtypes, including diffuse large B-cell lymphoma (DLBCL), Burkitt's Lymphoma (BL), Mantle Cell Lymphoma (MCL), Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma, Follicular Lymphoma (FL) and Nodal Marginal Zone Lymphoma (NMZL), and so forth [1]. DLBCL is one of the most malignant lymphomas in the world, accounting for nearly 40% of non-Hodgkin's lymphoma cases. It is highly heterogeneous from both morphological and clinical perspectives [2, 3]. Although great progress in diagnosis and treatment technologies has been made, many DLBCL patients still have poor prognosis [4]. Moreover, although several biomarkers have been identified to better classify and predict outcomes at diagnosis, the detailed mechanisms leading to occurrence and development of diffuse large B cell lymphoma have not been clarified [5]. MicroRNAs have been shown to have the potential for diagnosis and prediction of cancer [6-9]. Moreover, microRNA expression profiling could distinguish cancers based on both the cellular nature and developmental stage [10]. Therefore, the current study aimed to explore altered expression of miRNAs involved in DLBCL, providing more effective therapeutic opportunities.

MicroRNAs (*miRNAs*) are a group of naturally occurring, small (19 to 25-nucleotides), and non-coding RNAs that negatively regulating gene expression by binding to 3'-untranslated

Table 1. Clinicopathological information of the patients

	DLBCL (n=50)	BL (n=40)	Normal (n=25)
Age			
Range	22-84	25-81	23-85
Mean ± SD	57.94±14.04	56.70±13.42	57.84±15.42
Sex			
Male	27	19	14
Female	23	21	11

regions of target miRNAs [11-13]. Currently, about 10-30% of all human genes are regulated by microRNAs [14]. The latent importance of microRNAs in tumors has been discovered because of the aberrant expression of microRNAs that occurs in cancer-associated genomic regions, suggesting that microRNA dysfunction is a common feature in malignant tumors [15]. An increasing number of studies have discovered that aberrant expressed miRNAs are involved in a variety of human cancers and mediate in initiation, promotion, progression, and resistance to cancer chemotherapy [16-19]. Several studies have suggested that many miRNAs are highly expressed in DLBCL, such as miR-21, miR-106a-5p, miR-181a-5p, and miR-200c [20-22]. Furthermore, miR-21 expression profiles in tumor cells [8] and serum [9] have been proven to be associated with DLBCL patient prognosis. Microarray-based gene expression profiling is a potential method of investigating expression of miRNAs in DLBCL patients. Several groups have performed survival analyses on larger DLBCL patient cohorts by microarrays, confirming that miRNAs are associated with survival, including miR-21, miR-23a, and miR-222 [8, 23, 24]. However, studies concerning miRNA profiles of DLBCL have been scarce. Therefore, the present study was performed to detect miRNA expression profiles of DLBCL patients and normal lymphoid tissues using miRNA microarrays.

The current study reported miRNA expression profiles of 3 DLBCL tumors and 3 normal lymphoid samples, aiming to identify a DLBCLs specific miRNA signature. A total of 12 miRNAs were found to be differentially expressed in DLBCL. In addition, significantly aberrant miRNAs (*miR-181a-5p, miR-638, miR-1268b*) were validated using RT-qPCR in DLBCL, BL, and normal lymphoid samples. Present results revealed that the three miRNAs were more frequently expressed in DLBCL and BL, compared to normal lymphoid samples. Present data suggests that aberrant expression of miRNAs involved in DLBCL and BL may provide useful information in the search of future therapeutic targets.

Materials and methods

Clinical samples (discovery and validation cohorts)

For the current study, 50 DLBCL, 40 BL, and 25 normal paraffin-embedded tissues were obtained, between 2014 and 2016, from the Second Affiliated Hospital of Harbin Medical University, Heilongjiang Province, China. Of these, 3 pairs of DLBCL and corresponding nontumorous lymphoid tissues were collected, in parallel, for miRNA microarray analysis. Histopathologic slices were examined via H&E staining and FISH staining, then diagnosed separately by three pathologists. No patients received chemotherapy, radiotherapy, or immunotherapy before undergoing surgery. Sample collection was approved by the Ethics Committee of Harbin Medical University and written informed consent was obtained from each patient before tissue collection. Clinical information of the patients, including age and gender, are listed in **Table 1**. Patients with DLBCL had a mean age of 57.94±14.04 (22-84) years, while 27 patients (54%) were male. Patients with BL had a mean age of 56.70± 13.42 (25-81) years, while 19 patients (47.5%) were male. Normal controls had a mean age of 57.84±15.42 (23-85) years, while 14 patients (56%) were male.

RNA extraction and real-time quantitative PCR analysis (qRT-PCR)

Total RNA was isolated from DLBCL, BL, and normal lymphoid samples using the miRNAprep Pure FFPE Kit (Tiangen, Beijing, German, China), according to manufacturer protocol. Next, cDNAs of miRNAs were synthesized with miRcute Plus miRNA First-Strand cDNA Synthesis Kit (Tiangen, Beijing, German, China). RT reactions with miRcute Plus miRNA First-Strand cDNA Synthesis Kit contained 2.0 ug total RNA, 10 ul 2× miRNA RT Reaction Buffer, 2 ul miRNA RT Enzyme Mix, and RNase-Free ddH_2O_2 filled up to 20 ul in each reaction. The RT reaction was conducted under the following conditions: 42°C for 60 minutes, then 95°C

for 3 minutes. Afterward, the cDNA products from RT reaction were diluted 20 times. The purities and concentrations of cDNA were checked, spectrophotometrically, using NanoDrop ND-2000c (Thermo Fisher Scientific, Inc., Wilmington, DE). PCR was performed with miRcute Plus miRNA qPCR Detection Kit (Tiangen, Beijing, German, China) by an ABI PRISM 7500 fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR was carried out with 1.5 ul of the diluted products in 20 ul PCR reaction containing 10 ul 2× miRcute Plus miRNA Premix, 2 ul 50× ROX Reference Dye, 0.4 ul 10× miScript universal primer, and 0.4 ul 10× miScript primer assay. Amplification was performed as follows: 95°C for 15 minutes, followed by 45 cycles at 94°C for 20 seconds and 60°C for 34 seconds. All reactions were performed in triplicate. Expression of U6 was used as endogenous control for detection of miRNA expression profiles. The $2^{-\Delta Ct}$ method was used to quantify expression of miRNAs. Primers were used as follows: miR-181a-5p: 5'-AACAUUCAACGCUGUCGGUGAGU-3'; miR-638: 5'-AGGGAUCGCGGGCGGGUGGCGGCCU-3'; miR-1268b: 5'-CGGGCGUGGUGGUGGGGGUG-3'; U6: 5'-TGGTGAAGACGCCAGTGGA-3'.

miRNA microarrays

MicroRNAs from all RNA samples were labeled with Cy3 fluorescent dye and were turned into cDNA using RNA Agilent miRNA labeling Kit (Agilent, UK) and Spike Kit (Agilent, UK). Agilent microRNA Hybridization Kit (Agilent, UK) was then applied for hybridizing into Human miRNA Microarray, Release-21.0, 8×60K microarray slides (Agilent, UK) and scanning with Nimblegen MS200 array scanner. TIFF image files were obtained using Agilent Feature Extraction software to extract original data and quality control (QC) reports. Sample data, passing QC parameters, was subjected to quantile normalization and analyzed using GeneSpring GX (Agilent, UK) 13.0 software. MicroRNAs with *P*-values less than 0.05 and fold-change values of > 2 and < -2 are determined to be statistically significant.

Statistical analysis

Statistical analyses were conducted using SPSS software version 20 (IBM, New York, NY, USA) and GraphPad Prism 5.0. Hierarchical clustering analysis was used to describe miRNA expression profiles of 3 paired DLBCL and normal samples. Visualization of differentially-expressed miRNAs was conducted using R statistical language v.3.1.2. Moreover, miRNA expression is expressed as mean ± marked difference (X ± S) using the comparative ΔCt method. Comparisons between miRNA expression levels of the two different groups were conducted using Student's t-test. *P*-values were two-sided, with less than 0.05 indicating statistical significance.

Results

Differentially-expressed miRNAs profiling in DLBCL

To analyze worldwide miRNA profiles of diffuse large B-cell lymphomas, microarray analysis was conducted using a discovery set comprised of 3 normal tissues and 3 DLBCL samples. To check miRNA variations between DLBCL and normal tissues, this study evaluated data using scatter-plots. A total of 12 miRNAs were upregulated in DLBCL, while other miRNAs seemed to be same in tumorous and normal tissues in this study (**Figure 1A**). Microarray profiling also indicated that the 12 miRNAs were abnormally expressed, with *p* values < 0.05 (Student t-test) and at least a 2-fold change. A heat map representation of the 12 dysregulated miRNAs in DLBCL is shown in **Figure 1B**. Each of the 12 significantly differentially-expressed miRNAs were upregulated (*hsa-miR-181, hsa-miR-638, hsa-miR-328, hsa-miR-1268a, hsa-miR-1915-3p, hsa-miR-1268b, hsa-miR-1825, hsa-miR-4788, hsa-miR-4290, hsa-miR-3162-3p, hsa-miR-1202, hsa-miR-4649-3p*). Upregulated miRNAs are listed in **Table 2**. hsa-miR-4646-3p was upregulated the greatest, by almost 624-fold (P=3.88E-06).

Expression validation of selected miRNAs using qRT-PCR analysis in DLBCL and BL

B-cell NHL contains several different subtypes, including DLBCL, Burkitt's Lymphoma (BL), Mantle Cell Lymphoma (MCL), Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma, and Nodal Marginal Zone Lymphoma (NMZL) and so on. The current study validated results focusing mainly on DLBCL and BL. Three differentially-expressed miRNAs (miR-181, miR-638, and miR-1268) whose target genes were markedly enriched in important pathways and func-

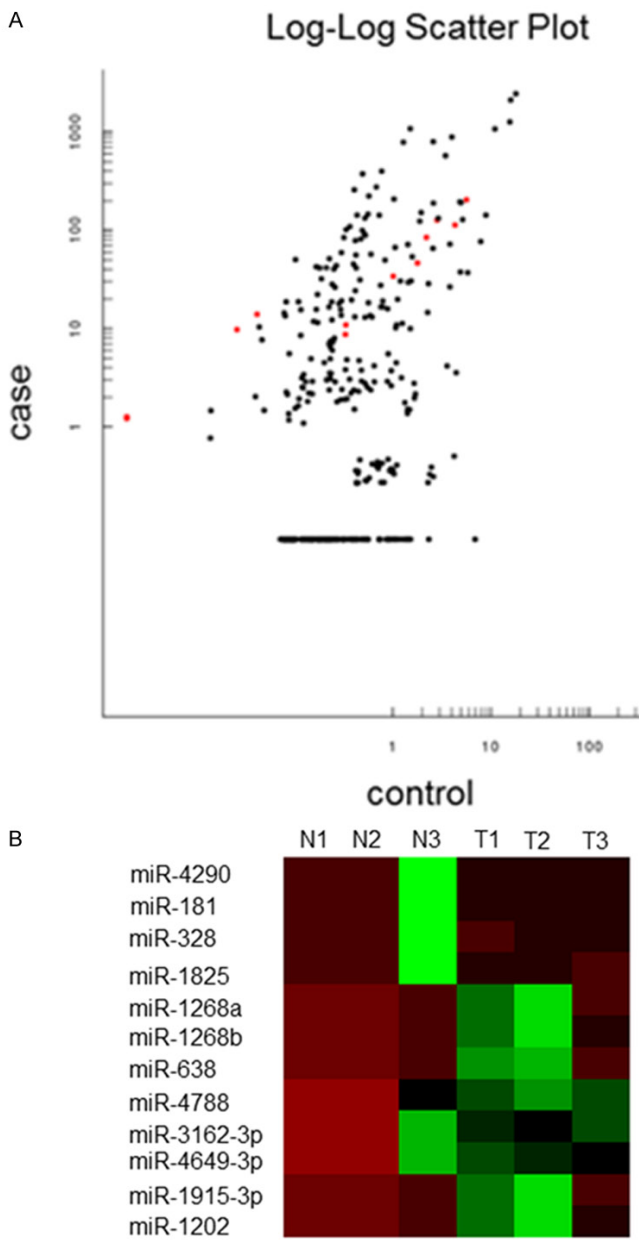

Figure 1. Analysis of microRNAs differentially expressed between DLBCL and normal controls. A: Scatter plot to show differences in miRNAs expression between DLBCL and normal control samples. The X axis and the Y axis represent the fluorescence intensity of the normal and the case group respectively. One dot shows one miRNA. Red represents high relative expression and green represents low relative expression. Marked black is no expression differences between the two groups. B: Hierarchical clustering for differentially expressed miRNAs in DLBCL (n=3) versus normal controls (n=3). Red indicates high relative expression and green indicates low relative expression. Missing values are indicated in gray.

tions were selected for further validation. Quantitative real time PCR (qRT-PCR) was used to validate microarray results among 50 DLBCL, 40 BL, and 20 healthy controls. Comparing DLBCL to control groups, *miR-181a-5p* ($P < 0.0001$), *miR-638* ($P=0.0001$), and *miR-1268b* ($P < 0.0001$) were found to have significantly increased expression levels (**Figure 2**). This study also confirmed that *miR-181a-5p* ($P=0.0058$), *miR-638* ($P < 0.0001$), and *miR-1268b* ($P < 0.0001$) were upregulated in BL, more than in controls. Results of qRT-PCR confirmed that three miRNAs, *miR-181*, *miR-638,* and *miR-1268,* in DLBDL and BL showed similar change patterns, as shown in microarray analysis (**Figure 2**).

Discussion

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma. MicroRNAs may provide a new management model of gene expression. Moreover, miRNA expression levels are closely associated with specific clinical features of tumors. They can be used to classify normal and tumor tissues and prognostic evaluation. MicroRNAs have a vital function in lymphomagenesis. However, the effects of miRNAs and their clinical roles in DLBCL have not been completely elaborated. Here, present results indicate that miRNA expression levels were clearly distinguished between DLBCLs and normal tissues. This study identified overexpression of *hsa-miR-181, hsa-miR-638, hsa-miR-328, hsa-miR-1268a, hsa-miR-1915-3p, hsa-miR-126-8b, hsa-miR-1825, hsa-miR-4788, hsa-miR-4290, hsa-miR-3162-3p, hsa-miR-1202, and hsa-miR-4649-3p* in DLBCLs. S Caramuta et al. used microarray analysis, finding that *miR-29b, miR-155,* and *miR-142-5p* were differentially-expressed in DLBCLs, compared to normal lymph nodes [25]. Charles H. Lawrie's

Table 2. Fold change and *P* values of differentially-expressed miRNAs with $P < 0.05$

miRNAs	Fold changes	*p*-value	Regulation
hsa-miR-181	623.5	3.88E-06	Up
hsa-miR-638	36.2	0.0491	Up
hsa-miR-328	377.0	0.0239	Up
hsa-miR-1268a	44.7	0.0498	Up
hsa-miR-1915-3p	26.1	0.0495	Up
hsa-miR-1268b	38.4	0.0448	Up
hsa-miR-1825	336.5	0.0423	Up
hsa-miR-4788	33.7	0.0486	Up
hsa-miR-4290	649.6	1.41453E-05	Up
hsa-miR-3162-3p	32.7	0.0389	Up
hsa-miR-1202	25.9	0.0419	Up
hsa-miR-4649-3p	26.4	0.0482	Up

research discovered that 40 and 20 microRNAs were up- and downregulated in DLBCLs, compared to controls (data not fully displayed) [23]. Lim et al. found that 63 miRNA-expression levels were increased and 39 miRNA-expression levels were decreased in DLBCLs (data not fully shown), especially expression of *miR-28-5p, miR-214-5p, miR-339-3p,* and *miR-5586-5p,* all associated with good prognosis [26]. Interestingly, most of the miRNA deregulation, in present results, has not been previously reported in DLBCL and/or other malignant tumors. Therefore, present results may provide some novel biomarkers for DLBCL. To further validate current results, real-time quantitative PCR detection of *miR-181, miR-638,* and *miR-1268* expression in diffuse large B-cell lymphoma and Burkitt's lymphoma was used. The latter is a common type of non-Hodgkin's lymphoma. Compared with the normal group, it was found that expression of *miR-181a-5p, miR-638,* and *miR-1268b* was elevated in diffuse large B-cell lymphoma and Burkitt's lymphoma. However, whether these molecules are abnormally expressed in all non-Hodgkin's lymphomas, or all lymphomas in general, remains to be elucidated.

In accord with current results, overexpression of miR-181 has been found in DLBCLs, compared to normal B-cells, in Emilia L Lim's study [26]. Some studies have reported that miR-181 dysregulated expression in many human cancers, such as acute myeloid-leukemia (AML), luminal breast cancer, non-small cell lung cancer, and oropharyngeal squamous cell carcinoma [27-30], but its roles in different cancers remain controversial. In Aleksandra Butrym's study, miR-181 expression was found significantly higher expressed in AML patients, compared to controls. Charles H. Lawrie et al. confirmed that ectopic expression of miR-181a and miR-181d promoted cell proliferation in luminal breast cancer. However, ectopic expression of miR-181a/d reduced anchorage independent growth and cancer stem cells phenotype in HPV16 transfected OSCC. Therefore, miR-181 plays a different role in carcinogenesis and tumor suppression in different tumors. Mechanisms concerning miR-181 in cancers are still relatively unknown. Chwan et al. suggested that miR-181 inhibited EphA5 and EphA7 expression, resulting in Nipah virus infection by increasing henipavirus-induced membrane fusion. There is also a research certifying that miR-181 activates Akt signaling by inhibiting INPP4B and PHLPP2 phosphatases to promote cancer cell entry S-phase in luminal breast cancer. Jian's study has proven that miR-181a-5p significantly decreased MMP-14 luciferase activity, thus miR-181a inhibits cancer cell metastasis by inhibiting MMP-14 expression [31]. Although Emilia L Lim results, along with present results, show that miR-181 was highly expressed and may function as an oncogene in DLBCL. However, the effects of miR-181 on DLBCL cell biological behavior and the potential mechanisms remain unclear. Future studies and discussion are necessary.

The current study explored expression of miR-638 in DLBCL, BL patients, and controls using qRT-PCR for the first time. Results suggest that expression levels of miR-638 were upregulated in DLBCL and BL patients, compared to healthy controls. Many studies have suggested that upregulation of miR-638 plays an important role in the development of melanoma, esophageal squamous cell carcinoma, and breast cancer [32, 33]. Ren Y et al. proved that miRNA-638 stimulates autophagy and malignant phenotypes of esophageal squamous cell carcinoma and breast cancer cells by suppressing DACT3 [32]. Animesh Bhattacharya et al. indicated that miR-638 promoted melanoma progression by inhibiting expression of p53 and p53 downstream target genes, resulting in decreased of apoptosis and autophagy. However,

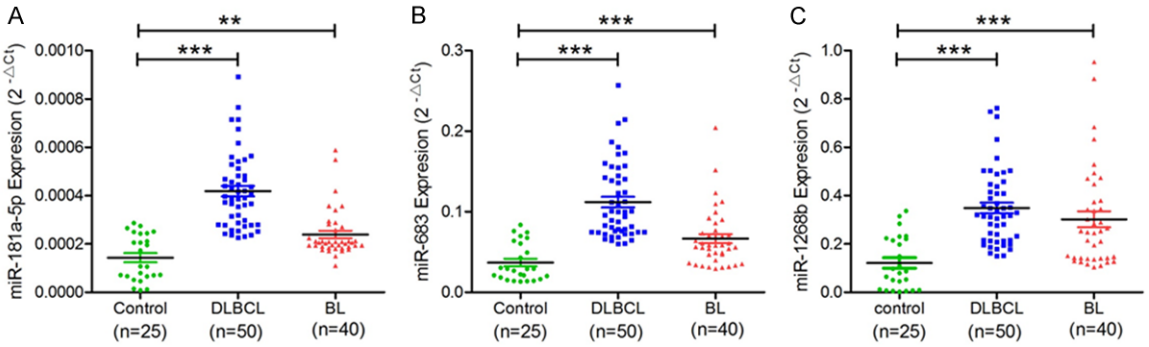

Figure 2. Validation of differentially expressed miRNAs by qRT-PCR in 50 DLBCL, 40 BL, and 25 normal samples. A: Relative expression levels of miR-181 in DLBCL, BL, and normal controls. B: Relative expression levels of miR-683 in DLBCL, BL, and normal controls. C: Relative expression levels of miR-1268 in DLBCL, BL, and normal controls. Statistical analyses were performed using unpaired Student's t-test. $P < 0.05$ indicates significance. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

other studies have suggested that miR-638 plays the role of tumor suppressor in cancer. One study confirmed that miR-638 restrains cell proliferation by inhibiting PIM1 genes in osteosarcoma [34]. Zhang's study demonstrated that miR-638 expression was reduced in hepatocellular carcinoma, suggesting that miR-638 refrained invasion and epithelial-mesenchymal transition by targeting SOX2 [35]. The different roles of miR-638 in different tumors may be due to actions on different target genes. Therefore, bioinformatics enrichment analysis and corresponding experiments should be conducted to explore that which target genes are regulated by miR-638 and which biological functions are affected in DLBCL and BL cells.

Present results demonstrated, for the first time, that miR-1268b is highly expressed in DLBCL and BL. However, the effects and mechanisms of miR-1268b on tumors remain unknown, as there have been very few studies concerning miR-1268 in tumors. Veronica Colangelo et al. suggested that miR-1268 was upregulated in facioscapulohumeral muscular dystrophy cells, compared to normal cells, indicating that miR-1268 may be involved in the regulation of cell proliferation [36]. A previous study showed that miR-1268 was repressed in peripheral blood mononuclear cell obtained from gestational diabetes (GDM) patients, suggesting that it may be involved in glucose metabolism in the human body [37]. However, further examination is necessary concerning the effects and mechanisms of miR-1268 on malignant cellular behavior in DLBCL and BL.

Many researchers have shown that many miRNAs regulate the expression and function of target genes, which participate in occurrence and development of DLBCL. Studies of clinical DLBCL cases have proven that miR-21 overexpression decreases expression of fork-head box protein O1 (FOXO1) and PTEN, resulting in the activation of PI3K/AKT/mTOR pathways and boosting more malignant biological behavior to DLBCL cells [38]. miR-155 promotes DLBCL cell growth by depressing expression of tumor suppressor gene phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1). Present results, however, should be verified in a larger population of DLBCL, with even more non-Hodgkin's lymphomas patients [39]. Overexpression of miR-125a/miR-125b directly decreases the activity of tumor necrosis factor alpha-induced protein 3 (TNFAIP3), thus activating NF-κB signaling pathways and participating in the progression of DLBCL [40]. Accordingly, overexpression of these oncomiRNAs is associated with decreased tumor suppressor gene activity and promotes proliferation, invasion, and metastasis in DLBCL, BL, and all kinds of non-Hodgkin's lymphomas. Therefore, GO and KEGG pathway enrichment analysis, clarifying mechanisms in the development of DLBCL and BL, is necessary.

In general, the current study determined miRNA expression profiles of FFPE tissues from DLBCL patients, and normal controls. Results confirmed the association between abnormal expression of 12 specific miRNAs and DLBCL patients. In particular, this study validated that miR-181a-5p, miR-638, and miR-1268b were

upregulated, possibly exerting potential oncogenic roles in DLBCL development. The current study may contribute to identification of potential diagnosis and prognostic biomarkers for DLBCL and BL patients. Further studies should be undertaken to investigate the impact of individual miRNAs in DLBCL patients, determining the effects on B-cell biology and lymphomagenesis. Many studies have shown that miRNAs have profound effects on expression of their target genes, suggesting that GO and KEGG Pathway enrichment analysis are necessary to elucidate their mechanisms in DLBCL and BL.

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Disclosure of conflict of interest

None.

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