INTRODUCTION

DLBCL is the most common type of adult lymphoma. Although DLBCL is highly aggressive, it is curable due to its high sensitivity to chemotherapy. However, a large number of DLBCL patients would have recurrence or refractory disease within the first 2-3 years after diagnosis and fail to achieve complete remission. Despite the advancements in therapeutic strategies, such as the liposomes doxorubicin and rituximab, which have greatly improved the treatment efficacy of DLBCL, the overall 5-year survival rate is still less than 50%. Therefore, further investigations on therapeutic targets involved in the pathogenesis of DLBCL might provide more effective treatment opportunities.

MicroRNAs (miRNAs) could modulate genes expression and have been shown to be critical regulators in tumorigenesis. In DLBCL, miRNAs were aberrantly expressed and validated as promising therapeutic targets. For example, miR-155 was overexpressed in DLBCL and predicted poor prognosis. Forced miR-144 expression could attenuate the proliferation and invasion of DLBCL cells. Recently, miR-216a functioned as a tumor suppressor in a variety of cancers, including pancreatic cancer, cervical cancer, lung cancer, and uveal melanoma. However, the role of miR-216a in DLBCL remains elusive.

YBX1 (Y box binding protein 1), which can bind to RNA or act as promoters of DNA through the highly conserved nucleic acid binding motif, is closely related to human malignant tumors. YBX1 was overexpressed in DLBCL tissues and predicted poor prognosis, metastasis, and formation of drug resistance in DLBCL. Moreover, miR-216a could target YBX1 to enhance the expression of transforming growth factor-stimulated clone 22, thus participating in collagen expression in kidney cells and diabetic nephropathy. However, whether YBX1 is involved in miR-216a-mediated DLBCL cell growth remains elusive.

This study validated the tumor-suppressive role of miR-216a in DLBCL and evaluated the potential target of miR-216a. These results highlighted the potential therapeutic role of miR-216a/YBX1 axis in DLBCL.
MATERIAL AND METHODS

Cell Culture

DLBCL cells (DB, SU-DHL-10, and SU-DHL-4) and normal B-cell line HMy2.CIR cells were obtained from DSMZ (Braunschweig, Germany). Cells were cultured in IMDM medium (Transgene, Beijing, China) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in an incubator.

Cell Transfection

Mimic and inhibitor of miR-216a, shRNAs targeting YBX1 (shYBX1#1: 5'-CAGGCGAAGGTTCCCACCTTA-3' and #2: 5'-CAAGAAGGTCATCGCAACGAA-3'), and the negative controls (Scramble, NC inhibitor, and shNC) were obtained from GenePharma (Shanghai, China). DB or SU-DHL-10 cells were transfected with mimic, inhibitor, and shRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 Assay

Twenty-four hours after transfection, DB or SU-DHL-10 (2 × 10^3 cells/well) cells were seeded in IMDM medium for another 48 h. Cells were incubated in 20 μL CCK8 solution (Dojindo, Tokyo, Japan) for 2 h before measurement of absorbance at 450 nm.

Bromodeoxyuridine Staining

Twenty-four hours after transfection, DB or SU-DHL-10 cells were incubated with 100 nM bromodeoxyuridine (Sigma-Aldrich) for 4 h. After fixing in paraformaldehyde, cells were incubated with 0.3% Triton X-100, 2M hydrochloric acid, and antipyronin, followed by blocking and incubating with the primary antibody against bromodeoxyuridine (Abcam, Cambridge, UK). Cells were then incubated with a secondary antibody before measurement of bromodeoxyuridine incorporation.

Transwell Assay

Twenty-four hours after transfection, DB or SU-DHL-10 cells were suspended in serum-free IMDM medium and then added into the upper chamber of the Matrigel-coated chamber (BD Biosciences, San Jose, CA, USA). Then, the IMDM medium with 20% serum was added into the lower chamber. Twenty-four hours later, cells in the lower chamber were fixed in 4% formaldehyde and stained with 0.5% methanol-prepared crystal violet before counting under a microscope.

Dual-Luciferase Reporter Assay

The region of miR-216a binding motif or the region of mutant binding motif in YBX1 (Sigma-Aldrich) for 4 h. After fixing in paraformaldehyde, cells were incubated with 0.3% Triton X-100, 2M hydrochloric acid, and antipyronin, followed by blocking and incubating with the primary antibody against bromodeoxyuridine (Abcam, Cambridge, UK). Cells were then incubated with a secondary antibody before measurement of bromodeoxyuridine incorporation.

qRT-PCR

RNAs were extracted from DLBCL cells (1 × 10^6) by RNAsimple Total RNA Kit (TIANGEN, Beijing, China). RNAs were reverse transcribed into cDNAs. qRT-PCR was performed by SYBR Green Master Mix (Solarbio, Beijing, China) with the primers listed below: miR-216a: F, 5'-TGTCGCAAATCTCTGCAGG-3', R, 5'-GAGCTCGTCCAGGTTCCACCTTA-3'; YBX1: F, 5'-GGAGTTTTGATGTTGTTTAGGAAGA-3', R, 5'-AAGGAGGAACCCACCGAGAAACTG-3'. GAPDH: F, 5'-GAGTCAACGCAGATTGGTGTCG-3', R, 5'-GACAAGCTCCCGTCTTCAG-3'; U6: F, 5'-CTCGTTTCCGAGAGCCACA-3', R, 5'-AACGCTTCAGAATTTGCGT-3'.

Western Blotting

Cell lysates (30 μg) were separated by 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. After blocking, membranes were immunoblotted with the following primary antibodies: YBX1 (1:2000; Abcam) and β-actin (1:3000; Abcam). Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam) before performing the procedure using Pierce ECL Western Blotting Substrate (Pierce Biotechnology, Waltham, MA, USA).

Statistical Analysis

Data were presented as mean ± standard deviation. Student's t test or one-way analysis of variance was applied to evaluate the data. A P-value < .05 was considered statistically significant. Dunnett t test was used in Figure 1 and paired t test was used in Figure 2, A–C, 3, 4B–E.

RESULTS

miR-216a Expression in DLBCL

To examine the expression of miR-216a in DLBCL cells, qRT-PCR analysis was performed in DB, SU-DHL-10, SU-DHL-4, and normal B-cell line HMy2.CIR cells. The results showed a significant reduction of miR-216a in DLBCL cells compared to HMy2.CIR cells (P < .001) (Figure 1), suggesting that miR-216a might be involved in DLBCL cell growth.

MiR-216a Suppressed DLBCL Cell Proliferation and Invasion

DB and SU-DHL-10 cells were transfected with miR-216a mimic to evaluate the role of miR-216a in DLBCL. DB (P = .003) and
SU-DHL-10 ($P = .002$) that were transfected with miR-216a mimic showed a higher expression of miR-216a than that transfected with Scramble (Figure 2A). Forced expression of miR-216a decreased the viability ($P = .010$ and $P = .035$) and SU-DHL-10 ($P = .010$ and $P = .041$) cells (Figure 2C), indicating the anti-proliferative effect of miR-216a in DLBCL. Moreover, invasion was also inhibited in DB ($P = .002$) and SU-DHL-10 ($P < .001$) cells transfected with miR-216a mimic (Figure 3), further suggesting the anti-invasive role of miR-216a in DLBCL. Therefore, these results revealed that miR-216a suppressed DLBCL cell proliferation and invasion.

**YBX1 Was a Target of miR-216a in DLBCL**

The TargetScan bioinformatics prediction algorithm (http://www.targetscan.org/vert_72/) suggested the potential binding sites between miR-216a and YBX1 (Figure 4A). The luciferase activity of YBX1-WT luciferase reporter vector was decreased by miR-216a mimic, whereas the activity of YBX1-MUT was not affected by the miR-216a mimic ($P = .001$) (Figure 4B). Moreover, to verify the regulatory effects of miR-216a on YBX1, DB cells were transfected with miR-216a mimic ($P = .002$) or inhibitor ($P = .006$), respectively (Figure 4C). Data from qRT-PCR (Figure 4D) and Western blot (Figure 4E) indicated that miR-216a mimic reduced YBX1 expression ($P = .005$ and $P = .010$), while miR-216a inhibitor enhanced the expression ($P = .007$ and $P = .010$), suggesting the functional role of miR-216a/YBX1 in DLBCL.

**Interference of YBX1 Suppressed DLBCL Cell Proliferation and Invasion**

DB and SU-DHL-10 cells were transfected with shRNAs targeting YBX1 (shYBX1#1 or #2) to evaluate the effect of YBX1 on DLBCL. DB and SU-DHL-10 cells transfected with shYBX1#1 or #2 showed a lower mRNA ($P < .001$) (Figure 5A) and protein ($P < .001$) (Figure 5B) expression of YBX1 than that of shNC transfected cells. Silence of YBX1 also decreased the viability (Figure 5C) of DB ($P = .021$ and $P = .005$) and SU-DHL-10 cells ($P = .006$ and $P = .003$), and suppressed DB ($P = .008$ and $P = .013$) and SU-DHL-10 ($P = .028$ and $P = .018$) cells proliferation (Figure 5D). Moreover, invasion of DB and SU-DHL-10 cells were also inhibited by shYBX1#1 or #2 ($P < .001$) (Figure 5E). Therefore, these results revealed that interference of YBX1 suppressed DLBCL cell proliferation and invasion.
DISCUSSION

Several miRNAs were related to the overall survival of patients with DLBCL and function as either tumor suppressor (MIRN21, MIRN34a) or oncogenic miRNAs (MIRN195, MIRN-LET7G). Overexpression or inhibition of miRNAs could inhibit cell growth and promote apoptosis, thus representing potential therapeutic strategies for the treatment for DLBCL. Previous studies showed that miR-216a suppressed oral squamous cell carcinoma metastasis through targeting eIF4B. Particularly, eIF4B was increased in DLBCL. Therefore, the role of miR-216a in DLBCL was determined.

Abnormal expressions of miRNAs have been found to predict clinical outcome and chemotherapeutic efficacy. Therefore, diagnostic or prognostic miRNAs in DLBCL could facilitate the therapies of patients with DLBCL. As previously reported, the significant hin of miR-216a-5p in colorectal cancer was associated with differentiation degree and various stages of tumor, suggesting that miR-216a-5p might be a diagnostic and prognostic biomarker for colorectal cancer. In this study, miR-216a was down-regulated in DLBCL cells, suggesting its possible diagnostic or prognostic role in DLBCL. However, further analysis on the relation between miR-216a expression and the clinicopathological parameters of DLBCL patients should be performed to confirm the role of miR-216a in DLBCL.

As a common tumor suppressor in various tumors, our results showed that miR-216a inhibited viability and proliferation, as well as suppressed the invasion of DLBCL cells. Epithelial to mesenchymal transition contributes to the metastasis of DLBCL through the regulation of epithelial and mesenchymal markers. Previous studies showed that overexpression of miR-216a-5p could silence the epithelial to mesenchymal transition way to suppress colorectal cancer metastasis. Therefore, the effect of miR-216a in the epithelial to mesenchymal transition of DLBCL should be investigated in further research. Moreover, considering a comprehensive delivery
system for the tumor-suppressor miRNAs, alternative therapies for miR-216a in DLBCL should also be further investigated.

MiR-216a-5p suppressed cell apoptosis in renal cell carcinoma, demonstrating its oncogenic role. MiR-216a could target tumor suppressors, phosphatase, and tensin homologues to promote the metastasis of endometrial cancer. A target gene involved in miR-216a-suppressed DLBCL was then determined. YBX1, which has the ability to bind to the Y box sequence (5’-CTGATTGG-3’), was the target gene of miR-216a in DLBCL. YBX1 could be activated by the MAPK/ERK pathway and then translocated into the nucleus to promote the progression of B-cell lymphoma. Nuclear localization of YBX1 demonstrated a worse prognosis than cytoplasmic YBX1 in DLBCL patients. Besides, ectopic expression of YBX1 promoted DLBCL cell proliferation through upregulation of cell cycle regulators. Here, miR-216a could decrease YBX1 expression, and YBX1 knockdown repressed the viability, proliferation, and invasion of DLBCL. YBX1 was also proved to be involved in miR-216a-suppressed colorectal cancer progression, and rescue experiments via YBX1 overexpression should be further performed to validate the role of miR-216a/YBX1 axis in DLBCL.

Moreover, serine 102 phosphorylation on YBX1 or other post-translational modifications, including acetylation, methylation, or ubiquitylation, have been reported to be implicated in tumor progression through the PI3K/Akt/mTOR pathway. Nuclear expression of YBX1 or other pathways-mediated modifications of YBX1 should be investigated in miR-216a-mediated DLBCL.

In conclusion, miR-216a directly targeted YBX1 and decreased YBX1 expression in DLBCL cells. MiR-216a repressed the viability, proliferation, and invasion of DLBCL. However, the subgroups of DLBCL were related to different clinical outcomes, distinct oncogenic activation mechanisms, and 5-year survival rates. Thus, the role of miR-216a in different subgroups of DLBCL needs to be further investigated.

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