A critical role of miR-144 in diffuse large B-cell lymphoma proliferation and invasion

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Abstract

MicroRNAs are endogenous noncoding RNAs that play important roles in a wide variety of biological processes such as apoptosis, development, aging and tumorigenesis. The BCL6 (B-cell lymphoma 6) transcriptional repressor has emerged as a critical therapeutic target in diffuse large B-cell lymphomas (DLBCL), but the mechanisms regulating BCL6 are still unclear. In the current study we screened the microRNA expression profiles in DLBCL specimens and cell lines by qRT-PCR, and found that the expression of miR-144 was significantly downregulated in DLBCL tissues and cell lines and negatively correlated with BCL6 expression. We further demonstrated that BCL6 was the direct target gene of miR-144, and miR-144 suppressed the expression of BCL6 via binding the 3’UTR of BCL6 mRNA. Biologically, forced expression of miR-144 significantly attenuated cell proliferation and invasion of OCI-Ly3 cells in vitro, and the tumor-suppressor effect of miR-144 was also confirmed using a xenograft mouse model in vivo. Taken together, our results revealed that miR-144 regulates BCL6 in DLBCL and provided rationales for developing strategies that target miR-144 as a therapeutic intervention for DLBCL.

Key words: miR-144, BCL6, DLBCL, proliferation, invasion
**Introduction**

As the most common subtype of non-Hodgkin’s lymphoma in adults, diffuse large B cell lymphoma (DLBCL) accounts for approximately 40% of diagnoses that come from the transformation of indolent follicular lymphoma (FL) (1). The technology of gene expression profiling has been used to stratify DLBCL into biologically meaningful and prognostically relevant subgroups (2). Although a portion of DLBCL patients can be cured, about 40% die of the disease (3), indicating the urgent need to develop more efficacious therapies.

As an evolutionarily conserved zinc finger transcription factor, B-cell lymphoma 6 (BCL6) protein is highly expressed in various human cancers, including malignancies in the lymphoid system (4). BCL6 protein is an important regulator of B lymphocyte growth and development (5, 6), and aberrant BCL6 protein expression is involved in the pathogenesis of many kinds of human hematologic malignancies, especially DLBCL (7-9). The checkpoint suppression properties of BCL6 are inherently pro-oncogenic and, accordingly, BCL6 is almost universally expressed in DLBCLs. In DLBCL, high expression of BCL6 is maintained, but the detailed molecular biological function is poorly understood.

MicroRNAs (miRNAs) are noncoding RNAs from 18–24 nucleotides in length that regulate gene expression in many biological processes, including development, proliferation, and differentiation (10). Recently, miRNAs have attracted attention because of their crucial roles in human diseases and as potential therapeutic targets. miRNAs participate in multiple physiological and pathophysiological processes of B cells, such as B-cell maturation and migration, interaction with immune niche cells, and the generation, production, and class-switching of immunoglobulins (11). An increasing body of evidence has demonstrated that miRNAs and BCL6 can target one another and mutually adjust their expression which are of great importance in the pathogenesis of various cancers, including breast cancer, prostate cancer, glioblastoma, and myeloma (12). A study reported that miR-127, as a regulator of
cellular senescence, directly targets BCL6 (13). However, little is known about the functions of the miRNAs that associate with BCL6 in DLBCL. Understanding these processes will provide new insights into treatment strategies for DLBCL patients.

MiR-144 was originally identified as an erythroid-specific miRNA required for the survival and maturation of erythroid lineage (14, 15). Ample studies have also revealed important roles for miR-144 in tumorigenesis and cancer treatment (16). In different types of cancers, down-regulation of miR-144 has been observed, such as osteosarcoma and mesothelioma, indicating miR-144 as a potential tumor suppressor (17, 18). Studies have revealed that miR-144 exerts its biological functions by targeting the 3’UTR region of the mRNAs of a number of oncogenes, such as ROCK1 (19), ZEB1 and ZEB2 (20), ADAM (21), TIGAR (22), and c-MET (23). However, whether miR-144 regulates BCL6 in carcinogenesis has not been investigated. In DLBCL cell lines, expression of miR-144 was rarely observed, which suggested that the interaction between miR-144 and BCL6 should be explored.

Methods and materials

Samples and Cells

All the non-Hodgkin’s lymphoma (NHL) and DLBCL patients' sample were from Department of Hematology, Affiliated Hospital of Weifang Medical College with informed consent, this study was approved by the research ethic committee of Weifang Medical College, and was executed in keeping with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments. Inclusion criteria for this study were age 18-65 years, primary B-cell lymphoma or T-cell lymphoma without clinical signs of other diseases and negative familial inherited diseases from 2012 to 2015. The immunohistochemical features and diagnosis of all cases were ascertained in accordance with the histopathology of H&E staining. Computed tomography (CT) scans and magnetic resonance imaging (MRI) were obtained in 31 cases of B-cell lymphoma patients and 14 T-cell lymphoma patients. Bone marrow examination was done and biopsy material was obtained by stereotactic
biopsy in all 58 cases.

Human DLBCL cell lines OCI-Ly3 and SUDHL2 cells, T-cell leukemia/lymphoma cell line Jurkat, and Burkitt lymphoma cell line, Raji, were purchased from the ATCC (Manassas, VA, USA) in 2014 with the original authentication and cultured in RPMI 1640 medium containing 10% fetal bovine serum. All cells were used within one year and passage number less than or equal to 10. The HEK293 cells were a gift of Dr. Tao Li, Associate Professor from Zhejiang Normal University, Department of Biology, and the HEK293 cells were only used for virus packaging and cultured in DMEM with high glucose. Cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C.

Plasmid constructions and Lentivirus packaging

Luc-BCL6 3’UTR (wild type) were commercially constructed by Ribobio (Guangzhou Ribobio, Guangdong, China), and the mutation of the miR-144–binding sites on Luc-BCL6 3’UTR was performed by TransGen (Beijing TransGen Biotech, Beijing, China). The lentivirus was purchased from GeneChem (GeneChem, China). Lentivirus carrying BCL6 or vector was packaged following the manufacturer's manual with a ratio of DNA to Lipo of 1:2. Lentivirus were packaged in HEK-293T cells and collected from the medium supernatant. Stable cell lines were established through infecting lentivirus into OCI-Ly3 cells and selected by 300 ng/ml puromycin for 3 weeks.

Oligonucleotide transfection

Scrambled and miR-144 mimics were obtained from Ribobio (Guangzhou Ribobio, Guangdong, China). Chemically synthesized miR-144 mimics are functional fragments sharing the same sequence as miR-144 to strengthen endogenous miR-144 expression. “Scramble” was used as the negative control. OCI-LY3 cells were transfected with miR-144 mimics or scrambled controls using Amaxa Nucleofector system and Amaxa Cell line Nucleofector Kit V (Lonza, NJ, USA), based on the manufacturer’s instructions.
Real-time RT-PCR
The real-time RT-PCR assay was done according to the manufacturer’s instructions. Briefly, RNAs from samples or cells were isolated using a Trizol protocol (Invitrogen, MA, USA) and DNase-digested (Ambion, MA, USA). cDNA was synthesized with M-MLV reverse transcriptase (Promega, WI, USA). ABI 7300 real-time RT-PCR system was employed for qRT-PCR assay with reagents iQ SYBR Green Supermix (Bio-Rad) and the appropriate primers. Primers for miR-144, -9, -93, -187, -124 and -10 were purchased from RiboBio (Guangzhou RiboBio, Guangdong, China). The primers were as follows: BCL6 (76bp): F: 5’-GGAGTCGAGACATCTTGACTGA-3’, R: 5’-ATGAGGACCGTTTTATGGGCT-3’; GAPDH (156bp): F: 5’-CGACCACTTTGTCAAGCTCA-3’, R: 5’-AGGGGTCTACATGGCAACTG-3’. The data presented for the qPCR are normalized cycle threshold (ΔCt) values. The formula ΔCt = Ct_BCL6 – Ct_GAPDH was used, in which a higher ΔCt value means less expression when the formula 2^−ΔΔCt is used.

Western blot
Cell lysates were prepared using 1× lysis buffer (Cell Signal, CA, USA); the same amounts of the cell lysate was subjected to 12% SDS PAGE; after electrophoresis, proteins were transferred onto a nitrocellulose membranes. The primary antibodies to BCL6 (sc-365618) and GAPDH (sc-365062) were purchased from Santa Cruz (Santa Cruz, CA, USA), and diluted at 1:500 for western blot; the second antibody was from the Jackson Lab (The Jackson Laboratory, MA, USA) and diluted at 1:2000 for western blot; the Pierce™ ECL Western Blotting Substrate was purchased from Life Technologies (NY, USA).

Luciferase assays
When OCI-Ly3 cells reached 70% confluence, cells were transfected with Scramble, miR-144 mimics and Luc-BCL6 3’UTR using VigoFect (Vigorous, China) according to the manufacturer’s instructions. Luciferase activity was calculated by the
Dual-Luciferase Reporter Assay System (Promega, WI, USA), and the values were normalized to Renilla luciferase values. The relative luciferase activity of the mimics group was normalized to that of the scramble groups, and represent data were from at least three independent experiments.

**Cell cycle and cell viability assay**

Cell cycle assay was analyzed using flow cytometry. Briefly, single cell suspension was fixed with 70% ethanol for 30 min at 4°C. RNA was degraded using RNase and DNA was labeled with propidium iodide (Sigma-Aldrich, MO, USA). Subsequently, DNA content was assessed by Epics xL flow cytometer (Beckman Coulter, UK). For cell viability assay, cells were seeded in 96-well plates at confluence of 2000 cells per well. The viability of the cells were calculated by a CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) based on the manufacturer's instruction at different indicated time points.

**Cell invasion assay**

Invasion was assayed using 24-well BD Matrigel invasion chambers (BD Biosciences, Cowley, UK) in accordance with the manufacturer's instructions. $5 \times 10^4$ cells per well were seeded in the upper well with DMEM without serum, the lower chamber well was DMEM medium containing 10% FBS. After 24 h incubation, non-invading cells on the top well were removed, and the bottom cells were fixed with 3% paraformaldehyde, subsequently stained with 0.1% crystal violet, and they were finally extracted with 33% acetic acid and detected quantitatively using a standard microplate reader (at 570 nm).

**Immunohistochemistry**

All human samples were obtained from the Department of Hematology, Affiliated Hospital of Weifang Medical College, with the informed consent. For immunohistochemical analysis of BCL6 expression in tissue samples, a mouse monoclonal antibody to BCL6 from Santa Cruz (sc-365618) (Santa Cruz
Biotechnologies, CA, USA) was used at a dilution of 1:100 according to immunohistochemistry procedure. Samples were stained with Haematoxylin (Sigma Aldrich, MO, USA) before mounting. Pictures were captured using an OLYMPUS BX51 digital microscope (Olympus, Japan).

Animal experiments
The animal experiments were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the animal studies were approved by the ethic committee of Weifang Medical College. All efforts were made to minimize suffering for the experimental animals, and the surgery was carried out under sodium pentobarbital anesthesia. Six SCID mice per group were injected subcutaneously into the left and right flank with $1 \times 10^7$ OCI-Ly3 cells resuspended in 200 μl PBS. Tumor sizes were measured every three days according to the formula: volume = $\frac{1}{2} \times$ length $\times$ width$^2$. After the last measurement mice were sacrificed humanistically and tumors were isolated.

Statistical analysis
Data are expressed as mean ± standard deviation (S.D.). Statistically significant differences were determined using one-way analysis of variance, SPSS version 21 software (IBM). Differences were considered to be statistically significant at P < 0.05.

Results
Expression of BCL6 in NHL
First we checked BCL6 expression in 39 cases of patients with B-cell lymphoma (including 8 cases of follicular lymphoma, 7 cases of Burkitt lymphoma, and 24 cases of DLBCL), and 19 cases of T-cell lymphoma (all 18 cases were peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) from 2012 to 2015 in the Department of Hematology, Affiliated Hospital of Weifang Medical College (Table 1), as well as the corresponding immortalized cell lines. We isolated the RNA from all these samples and detected BCL6 mRNA expression using real time PCR. Our results
showed that the ΔCt value (ΔCt = Ct_BCL6 - Ct_GAPDH) in the DLBCL group, which reflects the relative expression of BCL6, was significantly higher than that in other groups (Fig. 1A). Western blot assay in some representative samples of these subtypes also confirmed abundant BCL6 protein in the DLBCL patient’s sample, as well as in the OCI-Ly3 and SUDHL2 DLBCL cells; however, protein concentrations of BCL6 in follicular lymphoma, Burkett lymphoma and T-cell leukemia were relatively low compared with that in the DLBCL patients, and the same was found in the Raji cells and Jurkat non-Hodgkin's lymphoma cells (Fig. 1B). The amounts of BCL6 mRNA in the four cell lines were also consistent with protein amounts (Fig. 1C).

**Analysis and verification of miRNA target sites**

To identify which miRNAs target the **BCL6** gene, we first searched the miRNA prediction databases, TargetScan (Whitehead Institute for Biomedical Research, Cambridge, MA, USA), and found miR-144, -9, -93, -187, -124, and -10 were predicted as potential miRNAs that bind to **BCL6** gene mRNA. To determine functions of these miRNAs in the lymphoma, we examined the expression patterns of these miRNAs using qRT-PCR in different subtypes of B-cell lymphoma patients as well as cell lines, and found that expression of miR-144 presented significantly different expression pattern, but other microRNAs had no statistical difference (data not shown). To elucidate the detailed mechanisms of miR-144 in regulating BCL6 expression, we checked the potential targets using TargetScan and found a miR-144 conserved binding site including seven base pairs in the 3’UTR of **BCL6** gene mRNA (Fig. 2A). We overexpressed the miR-144 in OCI-Ly3 cells by transfecting the miR-144 mimics (Fig. 2B), and Western blot analysis showed that overexpression of miR-144 attenuated BCL6 expression in OCI-Ly3 cells compared with the scrambled control (Fig. 2C). A Luc-BCL6-3’UTR luciferase reporter was constructed to determine whether miR-144 directly target the 3’UTR of BCL6 gene mRNA and interrupts transcriptional activity. This reporter was cotransfected with miR-144 mimics (Mimics), for overexpression of miR-144, or the scrambled (Scr) control.
Luciferase assay results indicated that miR-144 overexpression significantly reduced luciferase activity in the wild-type Luc-BCL6-3’UTR reporter, but when the binding sites were mutated no significant differences were induced in the same cell line, suggesting BCL6 is a direct target of miR-144 (Fig. 2D).

**Negative correlation of miR-144 and BCL6 expression**

To explore whether expression of miR-144 and BCL6 correlated in DLBCL patients, BCL6 expression profiles were acquired by immunohistochemistry and western blot, and miR-144 expression was detected with qRT-PCR. All specimens were subjected to immunohistochemical staining for analysis of BCL6 expression. In the DLBCL lymphoma patients, the percentage of samples positive for BCL6 was over 70%, whereas the positive rate was only 14.2% for follicular lymphoma and 12.5% for Burkitt lymphoma. The T-cell lymphomas had a positive rate of 21%, as shown in a representative sample of BLBCL and other lymphoma in Fig. 3A (complete analytical data in Table 1). Some patients’ samples were tested for miR-144 expression by qRT-PCR and the result showed that miR-144 maintained low expression in DLBCL patients compared with other subtypes of B-cell lymphoma patients (Fig. 3B). In contrast, BCL6 expression was abundant in DLBCL patients, but very low in follicular lymphoma, Burkitt lymphoma, and T-cell lymphoma patients (Fig. 3C). These results indicated that miR-144 and BCL6 expression were negatively correlated in DLBCL patients.

**Effects of miR-144 on OCI-Ly3 cells proliferation and invasion**

To study the biological functions of miR-144 in DLBCL cells, we used OCI-Ly3 cells to examine the effect of miR-144 on cell proliferation and invasion. We performed CCK-8 assay and flow cytometry to verify the proliferation rate and cell cycle. 48 hours after transfection, the OCI-Ly3 cells transfected with miR-144 mimics proliferated much less than cells transfected with the scrambled and media controls ($P < 0.05$) (Fig. 4A). At the same time, the percentage of cells in the G0/G1 phase decreased from 53% to 71% with miR-144 overexpression (Fig. 4B). Typically,
expression of miR-144 was low in DLBCL cells, which might be related to OCI-Ly3 cell invasion. To study whether miR-144 indeed participates in DLBCL cell invasion, we also performed transwell assays to compare invasion ability at different miR-144 expression levels. We found that miR-144 overexpression in OCI-Ly3 cells resulted in suppressed invasion compared with scrambled and media controls (Fig. 4C). These data illustrated that miR-144 indeed has important biological roles in DLBCL cells.

**Exogenous BCL6 lacking a miR-144 binding site**

In order to determine whether miR-144 indeed inhibits proliferation and invasion through its target BCL6, we constructed a BCL6-expressing plasmid with its miR-144 binding sites mutated. When miR-144 mimics and BCL6 overexpression plasmids were cotransfected to OCI-Ly3 cells, miR-144 did not decrease the amount of protein exogenous BCL6 because BCL6 overexpression plasmid lacked miR-144 binding sites (Fig. 5A). CCK-8 assay and flow cytometry were employed, and although miR-144 was abundant in these cells, BCL6 overexpression still promoted cell growth and accelerated the cell cycle by augmented the percentage of cells in S phase (Fig. 5B, 5C). In the transwell assay, BCL6 overexpression enhanced cell invasion even though miR-144 was highly expressed (Fig. 5D). Without the interaction between miR-144 and BCL6 3’UTR, the effect of miR-144 was diminished, suggesting that miR-144 mediates proliferation and invasion through its transcriptional modulation on BCL6.

**Effects of miR-144 on lymphoma development in vivo**

To further confirm whether miR-144 could inhibit tumorigenesis in vivo, we used the SCID mice xenograft model to study the impact of miR-144 on DLBCL tumorigenesis and development. A lentivirus carrying miR-144 or Scramble was constructed and used to infect OCI-Ly3 cells, and these cells were selected in puromycin at 300 ng/ml for 3 weeks. We found that the initiation and the growth of tumor were significantly slower in the miR-144 overexpressing cells than the scramble control cells (Fig. 6A). After the last measurement mice were sacrificed and
tumors were isolated. The miR-144 overexpressing cells produced significantly smaller tumors than the Scramble cells (Fig. 6B). We further compared the expression of miR-144 and BCL6 in the tumor tissues isolated from the mice. The protein level of BCL and the RNA level of miR-144 in the tumors were negatively correlated (Fig. 6C, 6D). Therefore, our animal experiments confirm that miR-144 inhibits DLBCL growth in vivo.

**Discussion**

As a powerful inhibitor of senescence in primary mouse embryonic fibroblasts, BCL6 expression greatly extends the replicative lifespan of primary human B cells (24). Recently, BCL6 protein was also shown to be highly expressed in various human cancers including malignancies in the lymphoid system. For example, Chamdin *et al* reported that the expression of BCL6 in neuroblastoma is significantly associated with poor survival of the patients (25). In addition, BCL6 protein is expressed in the mammary epithelium in non-pregnant and early pregnancy animals (26), and in murine mammary epithelium, BCL6 overexpression prevents duct formation and apoptosis (27). However, BCL6 protein is highly expressed in breast cancer tissues, especially in high-grade ductal breast cancer (26, 27). Overall, the role of BCL6 protein in human cancers other than in the lymphoid system remains to be determined.

The properties of BCL6 as a therapeutic target come from its normal functions in the humoral immune system, where it plays important roles in enabling survival of germinal center (GC) B cells, from which DLBCL cells originate (28). After T-cell dependent antigen stimulation, B cells migrate within lymphoid follicles and form germinal centers (28). BCL6 is required for GC B-cell proliferation and tolerance to DNA damage, which occurs as a byproduct of immunoglobulin affinity maturation (28). Hence BCL6 participates in enabling and maintaining the GC B-cell phenotype (29). The majority of germinal center B cell-like (GCB) and activated B cell-like (ABC) type DLBCLs require and are hence “addicted” to BCL6 to maintain their proliferation and survival, reflecting its function in normal GC B cells and supporting
the notion that BCL6 is a broadly relevant therapeutic target for DLBCLs (30-32).

In the past decade, microRNAs have become the hot molecules for cancer biology, and many microRNA species have been shown to play important roles in tumorigenesis and cancer development. Based upon the abundant data accumulated during the past two decades, scientists began to argue for use of microRNAs as novel therapeutic targets for various malignancies (33). MiR-144 has been implicated in both oncogenic or tumor suppressor roles in different tumors, but its role in DLBCL is not yet clarified (34–36). Although research on the true biological relevance of miR-144 in cancer is still in its infancy, in this study we focused on the expression and function of miR-144 in DLBCL. The effect of miR-144 on DLBCL cells proliferation and invasion is probably achieved through BCL6 regulation. In the current study, we have acquired evidence to support this hypothesis: first, the expression of miR-144 is negatively correlated with BCL6 expression, in DLBCL tissues or cells; decreased BCL6 expression could be induced by overexpressing miR-144. Second, BCL6 is a direct target of miR-144, and miR-144 can indirectly inhibit lymphoma cells proliferation and invasion through targeting BCL6.

Conclusion

No prior report showed the direct involvement of miR-144 and BCL6 in DLBCL tumorigenesis, which warrants further investigation of this mechanism into DLBCL behavior. Although we should not rule out the involvement of other miRNAs whose target is BCL6, our results provide a proof of principle that miRNAs may be useful for future development of novel therapeutic strategies for DLBCL. Nonetheless, further investigations using animal models as well as more human samples must be done to validate our findings and to further elucidate the detailed mechanisms of miRNA functions in the treatment for lymphoma.

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Reference


PAX4-regulated miR-144/451 modulates metastasis by suppressing ADAMs expression. Oncogene. 2015;34(25):3283-95.


Table 1. Immunohistochemistry of BCL6 expression in non-Hodgkin’s lymphoma

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* Positive percentage is determined by number of strong staining samples over the total number of samples.
Figures Legends

Figure 1. Expression of BCL6 in B-cell Lymphoma

(A) Expression of BCL6 mRNA in different NHL samples, including 24 cases of diffuse large B-cell lymphoma (DLBCL), 8 cases of follicular Lymphoma (FL), 7 cases of Burkitt lymphoma (BL) and 19 cases of T-cell lymphoma (TCL). Primary specimens collected from patients were subjected to real time PCR assay and relative expression was assessed using ΔCt values (ΔCt= CtBCL6-CtGAPDH). The GAPDH gene served as the endogenous control. Data represent the results from three independent experiments.

(B) Western blots of representative BCL6 protein in some specimens from FL, BL, TCL, and DLBCL patients, as well as from two DLBCL cell lines and two other NHL cell lines. This experiment was repeated for three times. (C) Shown are relative expression of BCL6 mRNA in the Jurkat, Raji, OCI-Lc3, and SUDHL2 cells. The expression of BCL6 in the Jurkat cells was used to normalize the BCL6 expressions in the other cell lines, and GAPDH was used as the endogenous control. **P < 0.01 compared with the control. Data represent the results from three independent experiments.

Figure 2. BCL6 is a downstream target of miR-144

(A) Shown are putative miR-144-binding sites (position 53-63) on the BCL6 mRNA 3’UTR with potential complementary residues in bold. (B) Real time PCR of the miR-144 level in OCI-Ly3 cells after transfection of 100 pM of miR-144 mimics, Scramble (Scr), or mock control for 24 hours. Three independent experiments were averaged in this data. (C) Western blot analysis shows BCL6 protein in OCI-Ly3 cells transfected with 100 pM of Scramble (Scr) or miR-144 mimics (Mimics) for 48 hours. GAPDH served as a loading control. This experiment was repeated three times. (D) Luciferase vectors (luc-BCL6 3’UTR) carrying wide-type (WT) or mutated miR-144 binding sites on BCL6 mRNA 3’UTR (Mut) were co-transfected with miR-144 into OCI-Ly3 cells. Luciferase activities were measured and activity of renilla was used.
as internal control. **P < 0.01 compared with the control. Data represent the results of three independent experiments.

**Figure 3. Expression of BCL6 negatively correlated with miR-144 in DLBCL**

(A) Representative immunohistochemistry staining shows the BCL6 protein level in patients with DLBCL, FL, BL, and TCL lymphoma. Magnification, ×200. (B) Expression of miR-144 in different NHL samples, including 24 cases of diffuse large B-cell lymphoma (DLBCL), 8 cases of follicular Lymphoma (FL), 7 cases of Burkitt lymphoma (BL) and 19 cases of T-cell lymphoma (TCL). Primary specimens collected from patients were subjected to realtime PCR assay and the relative expression levels were analyzed using ΔCt values (ΔCt=Ctmir-144-CtU6). The U6 gene served as the endogenous control. *, P < 0.05 DLBCL compared with other groups. (C) Spearman correlation analysis showing relative expression of miR-144 and BCL6 in 24 DLBCL patient samples. An inverse correlation between miR-144 and BCL6 was observed (P < 0.001, R²=0.264).

**Figure 4. Effect of miR-144 on the growth and cell cycle of DLBCL cells**

(A) Shown is the proliferation curve of OCI-Ly3 cells measured by CCK-8 assay. After transfection of 100 pM of miR-144 Mimics (Mimics), Scramble (Scr) or mock control (Con) for 24 hours into OCI-Ly3 cells, cell proliferation was monitored at various time points. (B) Cell cycle analysis shows percentage of G0/G1, S, and G2/M phases in OCI-Ly3 cells transfected with miR-144 Mimics (Mimics), Scramble (Scr), or mock control (Con) measured by flow cytometry with propidium iodide (PI) staining. (C) Transwell analysis shows the effects of miR-144 on the migration of OCI-Ly3 cells. * P < 0.05 compared with the negative control. All data shown in this figure represent at least three independent experiments.

**Figure 5. Necessity of miR-144 binding site for miR-144 regulation of BCL6 expression.** (A) OCI-Ly3 cells cotransfected with vector or BCL6-overexpressing
plasmids lacking the miR-144 binding site (Bcl6 OE) with 100 pM of miR-144 mimics (miR-144 mimic) for 48 hours, after which the expression of the BCL6 protein was examined by Western blot. (B) Cell proliferation analysis was determined using CCK8 assay. (C) Cell cycle was analyzed using flow cytometry; (D) Cell invasion ability was explored by transwell analysis. \(*P < 0.05\) compared with the control. All data shown in this figure represent at least three independent experiments.

**Figure 6. Effects of miR-144 on tumor formation in the xenograft mouse model**

(A) Representative images of DLBCL tumor formation after 18 days in SCID mice bearing OCI-Ly3 cells with Scramble or with miR-144-overexpression (miR-144 OE). (B) Tumor volume changes in the mice bearing OCI-Ly3 cells with scramble control (Scramble) or with miR-144-overexpression (miR-144 OE) in a 18-day period. (C) Real-time PCR results for miR-144 and \(BCL6\) expression in the xenograft tissue from the scramble and miR-144 OE groups. (D) Western blot of BCL6 protein in the xenograft tissues from the above two groups (\(n = 6\) mice/group). The experiment was repeated three times. \(*P < 0.05\) compared with the negative control.
Figure 1
Figure 2
Figure 3
Figure 4


**Figure 5**

(A) Western blot analysis showing expression levels of miR-144 mimic, Bcl6 OE, Vector, BCL6, and GAPDH. 

(B) Graph depicting relative cell viability over time (0 h, 12 h, 24 h, 48 h, 72 h), comparing Empty, Vector, and Bcl6 OE groups. 

(C) Bar chart illustrating percentage distribution of cell cycle phases (G0/G1, S, G2/M) for Empty, Vector, and Bcl6 OE groups. 

(D) Graph showing relative invasion capability (fold) for Empty, Vector, and Bcl6 OE groups.
Figure 6
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