MicroRNA-21 regulates the viability and apoptosis of diffuse large B-cell lymphoma cells by upregulating B cell lymphoma-2

KE LIU[1], JINGXIA DU[2] and LINHAI RUAN[1]

[1]Department of Hematology, The First Affiliated Hospital and College of Clinical Medicine, Henan University of Science and Technology, Luoyang, Henan 471003; [2]Department of Pharmacology, Medical College, Henan University of Science and Technology, Luoyang, Henan 471003, P.R. China

Received November 18, 2015; Accepted December 19, 2016

DOI: 10.3892/etm.2017.5021

Abstract. Diffuse large B-cell lymphoma (DLBCL), one of the most frequently diagnosed non-Hodgkin lymphoma (NHL), is partly attributed to hereditary factors. MicroRNA-21 (miR-21) is an oncogenic substance that induces NHL and primarily targets tumor-suppressive molecules, such as B cell lymphoma-2 (Bcl-2). The present study explored whether Bcl-2, targeted by miR-21, would affect the development of NHL. Specimens were harvested from 55 patients with DLBCL who had undergone surgical treatment. Expression levels of miR-21 and Bcl-2 were evaluated through reverse transcription-quantitative polymerase chain reaction, immunohistochemistry and western blotting. Luciferase-reporter assays were performed to investigate the potential association between miR-21 and Bcl-2. MTT assays, flow cytometric analysis and caspase-3 activity assays were used to evaluate cell viability and apoptosis of DLBCL cells, respectively. Furthermore, statistical analysis was conducted using SPSS 19.0 software and the expression levels of miR-21 and Bcl-2 within DLBCL tissues were significantly upregulated when compared to those in normal tissues ($P<0.01$). As predicted by TargetScan, perfect base pairing was observed between the seed sequence of mature miR-21 and the 3' untranslated region of Bcl-2 mRNA. Dual luciferase reporter gene assays also revealed that miR-21 significantly facilitated the luciferase activity of Bcl-2 wild-type, with 61% upregulation ($P<0.01$) observed. MTT assays demonstrated that the viability of OCI-LY3 cells was decreased when cells were transfected with miR-21 inhibitor or Bcl-2 small interfering RNA and compared with those of control and negative control groups (all $P<0.05$). The apoptosis rate and caspase-3 activity level of the miR-21 group were 2.73±0.48 and 0.47±0.05, respectively, which were both significantly different from the groups with lower levels of miR-21 expression levels (all $P<0.01$). Since miR-21 may contribute to increased viability and decreased apoptosis of DLBCL cells through targeting Bcl-2, both Bcl-2 and miR-21 are likely to serve as effective targets for developing novel DLBCL treatments in the future.

Correspondence to: Dr Ke Liu, Department of Hematology, The First Affiliated Hospital and College of Clinical Medicine, Henan University of Science and Technology, 24 Jing Hua Road, Luoyang, Henan 471003, P.R. China
E-mail: keliul@163.com

Key words: diffuse large B-cell lymphoma, microRNA-21 B-cell lymphoma-2, proliferation, apoptosis

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a prevalent type of non-Hodgkin lymphoma (NHL) that occurs in developed countries (1) and the survival rate of untreated patients is only 50% for <1 year (1). Remarkable progress has been made in exploring the biological mechanism of DLBCL during the past decade, and results have indicated that environmental factors (2,3), dietary factors (4,5), genetic factors and clinical conditions may influence the risk of DLBCL (6,7). However, the pathology and mechanism of DLBCL still remains to be elucidated.

Major studies have started to focus on microRNA (miRNA), which are small non-coding RNAs composed of 20-22 nucleotides. miRNA have an important role in the lymphoid system, which is critical to the differentiation and malignant transformation of B-cells. A selection of miRNA function as regulators in oncogenic or tumor-suppressive pathways in lymphoma (8,9). Moreover, miR-21, miR-155 and miR-17-92 clusters have been acknowledged as oncogenic miRNA, which are believed to target tumor-suppressive molecules in various types of tumor, including glioblastomas, cholangiocarcinomas, lung cancer, breast cancer and colon cancer (10). Furthermore, overexpression of miR-21, miR-155 and miR-17-92 may be observed in lymphomas derived from B cells, T cells or natural killer cells (8,11,12). Notably, miR-21 has a vital role in regulating the chemosensitivity of DLBCL cells (13) and Bcl-2, a tumor-associated and anti-apoptotic molecule, has a key role in the chemoresistance of NHL and has been considered as a prognostic biomarker for DLBCL (14). However, the role of miR-21 in regulating the expression of Bcl-2 in DLBCL remains unclear, and there are no in-depth studies on the relationship between miR-21 and Bcl-2 in DLBCL.

The aim of the present study was to analyze the association between miR-21 and Bcl-2 expression levels in DLBCL cells. Furthermore, cell transfection, MTT, and flow cytometry

analysis were used to investigate whether miR-21 has an important role in modulating DLBCL cells.

Materials and methods

Patients and tissue samples. Specimens were obtained from 55 patients with DLBCL (30 men and 25 women) diagnosed using hematology at the First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology (Luoyang, Henan, China) between November 2012 and December 2014. The age of included patients ranged from 16 to 89 years, with a median age of 62. Histologic diagnoses were established according to the classification system outlined by the World Health Organization (15). According to the immune markers of cluster differentiation (CD)-10, Bcl-6, multiple myeloma oncogene-1 and Hans type principles (16), 55 patients with DLBCL were divided into germinal center B cell-like (GCB)-type (19 cases) and non-GCB type (36 cases) groups, with adjacent healthy lymph node tissues from the same patients as the control group. Tissue samples were frozen in liquid nitrogen immediately following surgery and stored at -80˚C. A portion of the tumor tissues were fixed in 10% formalin and embedded with paraffin. Sections of 4 μm thickness were examined with immunohistochemistry. The present study was approved by the Ethics Committee of the First Affiliated Hospital and College of Clinical Medicine, Henan University of Science and Technology, and all participants gave their written informed consent.

Detection of miR-21 with reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from tissue samples was extracted using TRIzol reagent and purified using a miRNeasy Mini Kit (Qiagen GmbH; Hilden, Germany). Genomic DNA was removed with DNase treatment and quantified using NanoDrop ND-2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A ratio of OD_{260}/OD_{280} between 1.7 and 2.1 indicated a higher purity of RNA which was considered to be satisfactory for follow-up experiments. The Omniscript reverse transcription kit (Qiagen GmbH) was used to reverse transcribe total RNA into cDNA according to the manufacturer's protocol. Expression levels of miR-21 were detected using the QuantiTect SYBR Green PCR Kit (Qiagen GmbH). The primer sequences for miR-21 were: Sense, 5'-GCGCGTCGTGAAGCGTTC-3'; antisense, 5'-GTGCAGGGTCCGAGGT-3'. The total reaction volume was 10 μl, containing the following: MirVana 56 RT buffer (2 μl), miR-21 RT primer (1 μl), ArrayScript Enzyme Mix (0.4 μl), total RNA (0.2 μl), and H_2O (6.4 μl). The cycling conditions were as follows: 95˚C for 30 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. All assays were repeated three times. The relative expression quantity of miR-21 was calculated using the $2^{-\Delta\Delta Cq}$ method (17) and normalized to the expression of U6 snRNA.

Detection of Bcl-2 with immunohistochemistry. Bcl-2 expressed in DLBCL tissues was analyzed using immunohistochemical streptoavidin-biotin peroxidase. Tissues were fixed with 4% formalin at 4˚C for 12 h. Formalin-fixed paraffin-embedded tissues obtained from 55 DLBCL tissues and 12 normal lymphoma tissues were cut into 4-μm slices. Subsequently, the following procedures were applied: Conventional dewaxing, graded ethanol dehydration, antigen retrieval were performed, and membranes were incubated with 3% hydrogen peroxide to block endogenous peroxidase (4˚C for 10 min), and normal sheep serum (Cappel Laboratories, Cochranville, PA, USA) to block the tissue sample (27˚C for 20 min). Primary mouse anti-human Bcl-2 monoclonal antibody (1:500; cat. no. IS61430, Dako, Glostrup, Denmark) was applied, incubated at 4˚C overnight and stored at room temperature for 20 min. Membranes were washed three times with Tris-buffered saline with Tween 20 (TBST) for 10 min and subsequently incubated with the secondary antibody (anti-IgG; 1:2,000 dilution; cat. no. P0448, Dako) that was labeled with biotin (Dako), and the streptomycin-avidin that was labeled with horseradish peroxidase (Dako) for 30 min at room temperature. Membranes were washed again with TBST three times for 10 min, staining was performed using diaminobenzidine and slices were counterstained with hemalum. Phosphate-buffered saline instead of a primary antibody was considered as a negative control (NC) and a known positive antibody anti-CD38 (1:100; cat. no. TA353695, Origene Technologies, Inc., Rockville, MD, USA) was set as a positive control. Moreover, the product of staining intensity (3, brown; 2, yellow; 1, light yellow; and 0, colorless) and the percentage of positive cells (4, >75%; 3, 51-75%; 2, 26-50%; 1, 6-25%; and 0, <5%) were complied with the integral calculation method for Bcl-2. Cells were randomly selected from five high power fields under a light microscope (magnification, x400) in each slice and 100 cells were counted in each field. Based on the two types of scores, the integral levels were evaluated as: Negative (-), 0 points; weak positive (+), 1-2 points; positive (++), 3-5 points; strongly positive (+++), >5 points. The slides were independently evaluated by two blinded pathologists.

miRNA target prediction and 3' untranslated region (UTR) luciferase-reporter assay. MiRNA targets were predicted using the TargetScan database version 7.1 (http://www.targetscan.org/vert_71/). Wild-type and mutant-type Bcl-2 3'UTR luciferase reporter vectors were constructed. miR-21 mimics or control were co-transfected with constructed wild-type or mutant-type luciferase reporter vector into DLBCL OCI-LY3 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific Inc.). The pRL-TK control vector (Promega Corporation; Madison, WI, USA) was transfected and served as a control. Subsequently, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (E1910; Promega Corporation) following cell transfection for 48 h.

Cell culture and cell transfection. OCI-LY3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were cultured in an incubator containing 5% CO_2 at 37˚C and the OCI-LY3 cell line was provided by the Chinese Academy of Sciences (Guangzhou, China). Cells were divided into five different groups: Control, NC, miR-21 mimics, miR-21 inhibitor, and Bcl-2 siRNA groups. Cells without any treatment were in the control group, while the other four groups were transfected with negative control (empty vector), miR-21 mimics, miR-21 inhibitor and Bcl-2 siRNA, respectively. The corresponding vectors were purchased from Shanghai

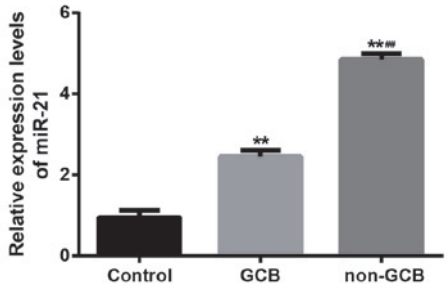

Figure 1. Relative expression levels of miR-21 in the control and diffuse large B cell lymphoma tissues detected by reverse transcription-quantitative polymerase chain reaction. Data are presented as mean + standard deviation (n=3). **P<0.01 vs. the control group, $^{\#\#}$P<0.01 vs. the GCB group. miR-21, microRNA-21; Control, normal lymph node tissues; GCB, germinal center B cell-like tissues; Non-GCB, non-germinal center B cell-like tissues.

GenePharma Co., Ltd (Shanghai, China). Cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific Inc.) and cultured in an incubator containing 5% CO_2 at 37˚C. Complete medium was replaced every 6 to 8 h until the culture process was completed.

MTT assay. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assays were used to evaluate cell viability. Transfected cells, which were washed twice with PBS, were cultured to a confluence of 80%, digested with trypsin and constructed into cell suspensions and the number of cells were counted manually. OCI-LY3 cells were inoculated into 96-well plates at a cell density of 3-6x10^3 cells/well. Six wells were replicated. Cells were detected following transfection for 24, 48, 72, and 96 h, respectively. MTT (20 μl; 5 mg/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added and the cell culture was sustained for 4 h at 37˚C in an incubator with 5% CO_2. Subsequently, DMSO (150 μl) was added into each well and the cells were lightly shaken for 10 min to dissolve the crystals. Samples were detected using a microplate reader (SpectraMAX Plus; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 490 nm. An MTT curve was drawn with the absorbance value as the vertical axis and the time interval as abscissa.

Flow cytometric analysis. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kits (BD Biosciences, San Jose, CA, USA) were used to evaluate the apoptosis of OCI-LY3 cells. Following 48-h transfection, cells were washed twice with cold PBS and re-suspended with binding buffer to a density of 0.5-1x10^6/ml. Subsequently, cell suspensions (100 μl) were incubated with 5 μl of annexin V-FITC and PI in the dark for 15 min at room temperature. Binding buffer (400 μl) was added to each tube and cells were analyzed using flow cytometry (Beckman FC 500 MCL/MPL; Beckman Coulter, Inc., Brea, CA, USA).

Detection of caspase-3 activity. Caspase-3 activity was detected using the caspase colorimetric assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), following cell transfection for 48 h. Cells were lysed in lysis buffer on ice for 20 min in order to detect the activity of caspase-3. Following centrifugation at 28,341 x g for 5 min at 4˚C, supernatants were incubated with the caspase substrate in the reaction buffer at 37˚C for 4 h. Samples were detected with a SpectraMAX Plus microplate reader at the wavelength of 405 nm. Relative caspase-3 activity was calculated as the percentage of A405 values in the experimental samples over those in the control groups.

RT-qPCR for detecting the expression levels of Bcl-2 mRNA. The extraction of cellular total RNA was conducted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was reverse transcribed from RNA using the Omniscript RT kit (Qiagen GmbH). qPCR detection of Bcl-2 mRNA was performed using the QuantiTect SYBR Green PCR Kit (Qiagen GmbH). Primers used for Bcl-2 (Invitrogen; Thermo Fisher Scientific Inc.) were as follows: Bcl-2 sense, 5'-CTGTGCTGCTATCCTGC-3' and antisense, 5'-TGCAGCCACAATACTGT-3'. Relative expression levels of Bcl-2 were calculated using the $2^{-\Delta\Delta Cq}$ method (17) and β-actin was set as the corresponding control.

Western blotting assay. Bcl-2 expression was detected by western blotting. Cellular proteins were extracted after 48-h transfection and the bicinchoninic acid method was used to evaluate the protein density. Furthermore equal quantities of protein (50 μg) from each group were loaded and separated by 10% SDS-PAGE, transferred onto polyvinylidene fluoride membranes, and blocked with 5% non-fat milk for 1 h at room temperature. Membranes were incubated with primary mouse anti-human Bcl-2 monoclonal antibody (1:500) and GAPDH antibody (1:1,000; cat. no. 5174, Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. Furthermore, membranes were washed three times with TBST for 10 min each and incubated with horseradish-peroxidase-linked anti-IgG (1:2,000; Origene Technologies, Inc.) at room temperature for 1 h. Membranes were washed again with TBST three times (10 min each) and signal detection was performed using a Super Enhanced Chemiluminescence Plus Detection Reagent (Applygen Technologies Inc., Beijing, China). The samples were quantified using Lab Works software version 4.5 (Mitov Software, Moorpark, CA, USA) with GAPDH as an internal control.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) and P<0.05 was considered to indicate a statistically significant difference. Significant differences in continuous data (mean ± standard deviation) were analyzed using the analysis of variance with Student Newman-Keuls post-hoc tests for comparisons between groups, and differences in continuous data between two groups were analyzed using unpaired Student's t-tests. Furthermore, results of immunohistochemistry for Bcl-2 protein were analyzed by the rank sum test. The association between miR-21 and Bcl-2 protein expression was analyzed using the Spearman rank correlation.

Results

miR-21 and Bcl-2 protein expression levels in DLBCL clinical specimens. RT-qPCR was used to evaluate the expression level of miR-21 in 19 cases with GCB-DLBCL, 26 cases without GCB and 20 normal lymph node tissues (Fig. 1). The expression

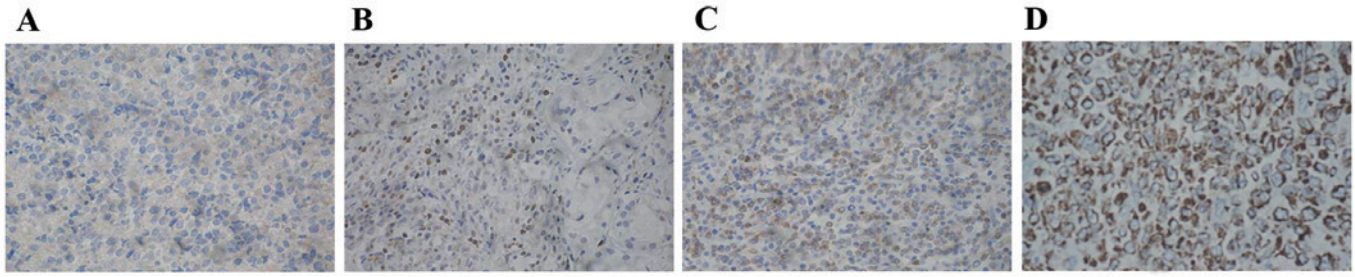

Figure 2. Relative expression levels of Bcl-2 in clinical specimens were detected via the immunohistochemical streptoavidin-biotin peroxidase method (magnification, x400). (A) Bcl-2- cells; (B) Bcl-2+ cells; (C) Bcl-2++ cells; and (D) Bcl-2+++ cells. Bcl-2, B-cell lymphoma-2. Staining intensity was indicated as follows: 3, brown; 2, yellow; 1, light yellow; and 0, colorless. The percentage of positive cells (4, >75%; 3, 51-75%; 2, 26-50%; 1, 6-25%; and 0, <5%) were complied with the integral calculation method for Bcl-2. OCI-LY3 cells were randomly selected from five high power fields (magnification, x400) in each slice and 100 cells were counted in each field. Based on the two types of scores, the integral levels were evaluated as: Negative (-), 0 points; weak positive (+), 1-2 points; positive (++), 3-5 points; or strongly positive (+++), >5 points.

Table I. Expression of Bcl-2 in diffuse large B cell lymphoma and control groups.

		Bcl-2					
Groups	N	-	+	++	+++	Z-value	P-value
Control	12	12	0	0	0	-2.331	0.02[a]
GCB	19	12	4	2	1	-2.053	0.04[b]
N-GCB	36	13	10	5	8	-3.576	<0.01[c]

Bcl-2, B cell lymphoma-2; GCB, germinal center B cell-like tissues; N-GCB, non-germinal center B cell-like tissues. [a]Control vs. GCB group. [b]GCB vs. N-GCB group. [c]Control vs. N-GCB group.

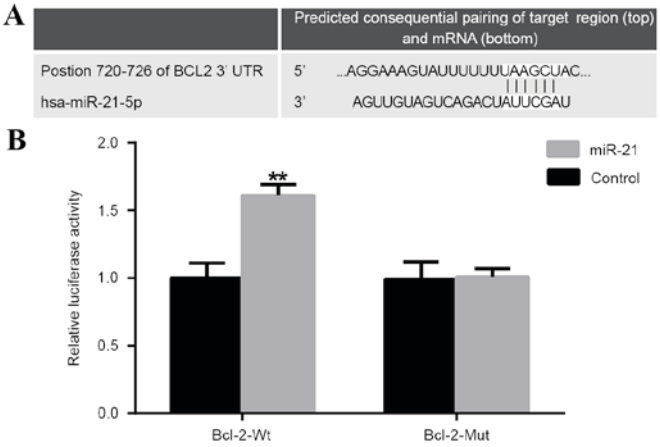

Figure 3. Bcl-2 is a target gene of miR-21. (A) Binding of miR-21 to Bcl-2 3'-UTR was predicted by TargetScan. (B) Dual luciferase reporter gene assay revealed miR-21 significantly increased the luciferase activity of Bcl-2 wt 3'UTR. Data are presented as the mean + standard deviation (n=3). **P<0.01 vs. the corresponding control group. Bcl-2, B-cell lymphoma-2; miR-21, microRNA-21; 3'-UTR, three prime untranslated region; wt, wild-type.

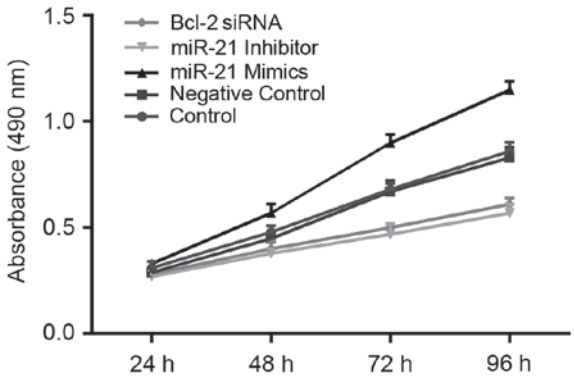

Figure 4. Viability of OCI-LY3 cells was assessed by MTT assay. OCI-LY3 cells were transfected with control, negative control, miR-21 mimics, miR-21 inhibitor or Bcl-2 siRNA, respectively, for 48 h. Results are presented as the mean ± standard deviation from three independent experiments. Bcl-2, B-cell lymphoma-2; miR-21, microRNA-21; siRNA, small interfering RNA.

of miR-21 in DLBCL tissues was significantly increased when compared to that in normal tissues (P<0.01) and the expression level of miR-21 in non-GCB DLBCL tissues was significantly higher than GCB-DLBCL tissues (P<0.01; Fig. 1). In addition, positive Bcl-2 expression was predominantly detected in the cell cytoplasm or cell membrane with yellow granules. Bcl-2-positive cells appeared to be focally gathered or unevenly distributed (Fig. 2). Bcl-2 expression levels were significantly increased in non-GCB DLBCL tissues when compared with normal tissues (P<0.01) and GCB-DLBCL tissues (P<0.05); the positive expression of Bcl-2 in GCB-DLBCL tissues was significantly increased when compared with normal tissues (P<0.05; Table I).

Spearman correlation analysis suggested that there was a positive correlation between miR-21 and Bcl-2 expression levels in both GCB-DLBCL (r_s=0.528, P=0.02) and non-GCBDLBCL tissues (r_s=0.708, P<0.01), whereas no significant correlation between miR-21 level and Bcl-2 protein level was detected in normal tissues.

Targeting Bcl-2 by miR-21. A putative conserved binding site for miR-21 at nucleotide position 720-726 of human Bcl-23'UTR was predicted using TargetScan. Perfect base pairing was observed between the seed sequence of mature miR-21 and the 3'UTR of Bcl-2 mRNA (Fig. 3A). Dual luciferase reporter gene assays revealed that miR-21 significantly promoted the luciferase activity of Bcl-2 wild-type with an upregulation of 61% (P<0.01); however, there was no significant effect on the luciferase activity of Bcl-2 mutant-type 3'UTR (Fig. 3B).

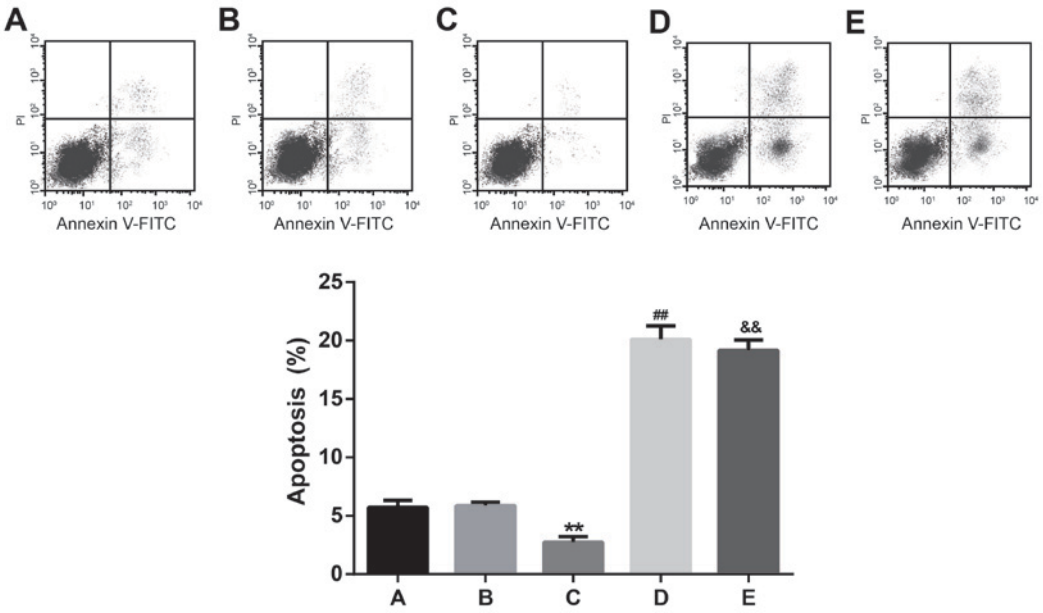

Figure 5. Apoptosis of OCI-LY3 cells was analyzed using flow cytometry. OCI-LY3 cells were separately transfected with control, negative control, miR-21 mimics, miR-21 inhibitor or Bcl-2 siRNA for 48 h. The percentage of apoptosis was detected by flow cytometry and shown in the bar graph. Results are presented as the mean + standard deviation of three independent experiments. **P<0.01 vs. the control, negative control, miR-21 inhibitor and Bcl-2 siRNA groups; ##P<0.01 vs. the control, negative control and Bcl-2 siRNA groups; &&P<0.01 vs. the control and negative control groups. Bcl-2, B-cell lymphoma; miR21, microRNA-21; siRNA, small interfering RNA.

Collectively, these findings indicated that Bcl-2 was likely to be a direct target for miR-21.

Proliferation of DLBCL cells. Results of MTT assays suggested that decreased proliferation of OCI-LY3 cells was observed in groups transfected with miR-21 inhibitor and Bcl-2 siRNA for 48 h when compared with the control and NC groups (all P<0.05). This trend was more pronounced as the length of time extended. miR-21 inhibitor appeared to have a stronger ability than Bcl-2 siRNA with respect to the inhibition of cell proliferation; however, this distinction did not reach statistical significance. The cell number of the control group at each time point was not significantly different from that of the NC group. Compared with the other four groups, the proliferation capacity of OCI-LY3 cells significantly increased after the transfection of miR-21 mimics for 48 h (all P<0.05; Fig. 4).

Apoptosis assay. Results of flow cytometry analysis indicated that the apoptosis percentages of cells transfected with miR-21 inhibitor and Bcl-2 siRNA were 20.10±1.16 and 19.15±0.91%, with no significant difference observed. However, the apoptosis percentages of miR-21 inhibitor and Bcl-2 siRNA groups were significantly higher than the miR-21 mimic, control and NC groups (P<0.01; Fig. 5). Furthermore, there was no significant difference observed in the apoptosis percentage between the control (5.71±0.62%) and NC (5.86±0.32%) groups. The apoptosis rate (mean ± standard deviation) of the miR-21 mimic group was 2.73±0.48%, which was significantly lower than those of the other four groups (P<0.01; Fig. 5). These findings indicated that miR-21 may inhibit the apoptosis of OCI-LY3 cells and the downregulation of miR-21 or Bcl-2 may increase the apoptotic ability of OCI-LY3 cells.

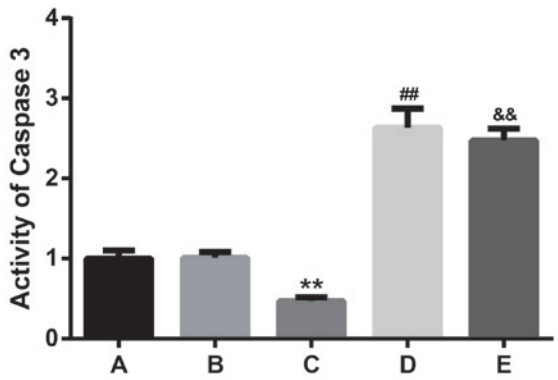

Figure 6. Caspase-3 activity in cells analyzed after transfection with the control, negative control, miR-21 mimics, miR-21 inhibitor, or Bcl-2 siRNA for 48 h. Results presented are the mean + standard deviation of three independent experiments. **P<0.01 vs. the control, negative control, miR-21 inhibitor and Bcl-2 siRNA groups; ##P<0.01 vs. the control, negative control and Bcl-2 siRNA groups; &&P<0.01 vs. the control and negative control groups. Bcl-2, B-cell lymphoma; miR21, microRNA-21; siRNA, small interfering RNA.

Caspase-3 activity assay. The caspase-3 activity in cells transfected with miR-21 mimics was 0.47±0.05, indicating a significant difference when compared with the other four groups (all P<0.01; Fig. 6). Caspase-3 activity levels for cells transfected with miR-21 inhibitor and Bcl-2 siRNA were 2.6±0.24 and 2.47±0.15, with no significant difference. However, the caspase-3 activities in miR-21 inhibitor and Bcl-2 siRNA groups were significantly increased compared with the control, NC and miR-21 mimic groups (P<0.01; Fig. 6). These results suggest that miR-21 may restrain the activity of caspase-3 and the downregulation of miR-21 or Bcl-2 may increase the caspase-3 activity.

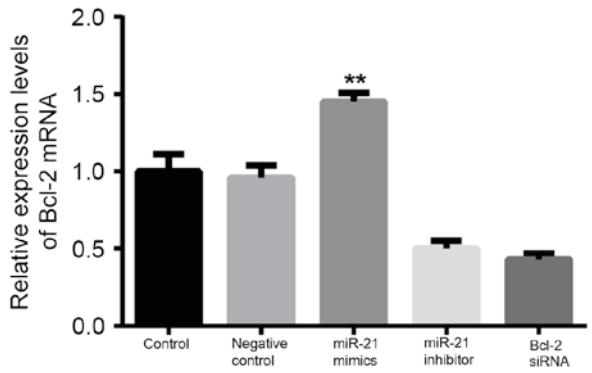

Figure 7. miR-21 increases Bcl-2 mRNA expression. OCI-LY3 cells were transfected with the control, negative control, miR-21 mimics, miR-21inhibitor, or Bcl-2 siRNA for 48 h. Bcl-2 mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction. Results presented are the mean + standard deviation of three independent experiments. **P<0.01, vs. the control, negative control, miR-21 inhibitor and Bcl-2 siRNA groups. Bcl-2, B-cell lymphoma; miR21, microRNA-21; siRNA, small interfering RNA.

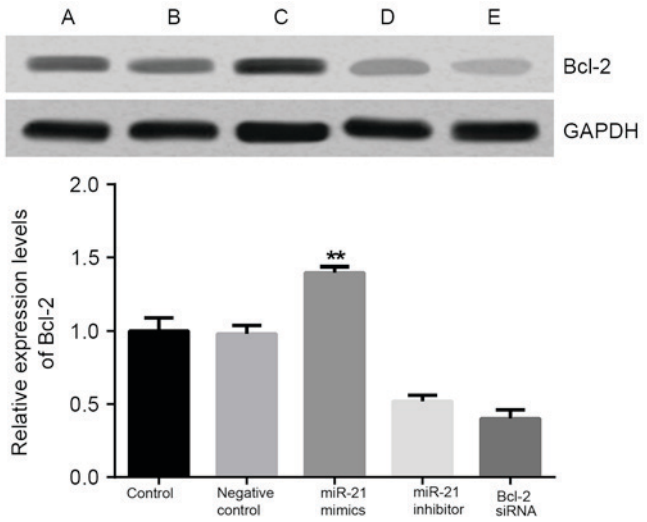

Figure 8. Increased expression of Bcl-2 was induced by miR-21 and detected by western blot assay. OCI-LY3 cells were transfected with (A) control, (B) negative control, (C) miR-21 mimics, (D) miR-21 inhibitor or (E) Bcl-2 siRNA. Results indicate the mean + standard deviation of three independent experiments. **P<0.01 vs. the control, negative control, miR-21 inhibitor and Bcl-2 siRNA groups. Bcl-2, B-cell lymphoma; miR21, microRNA-21; siRNA, small interfering RNA.

Bcl-2 mRNA and Bcl-2 protein expression levels in DLBCL cells. Results from RT-qPCR and western blotting demonstrated that the expression levels of Bcl-2 mRNA and Bcl-2 protein were significantly decreased when compared to the control and NC groups after 48 h transfection with the Bcl-2 siRNA or miR-21 inhibitor (P<0.01; Figs. 7 and 8, respectively). No significant difference in Bcl-2 mRNA and Bcl-2 protein expression levels were indicated between the control and NC group (P>0.05). Bcl-2 expression levels in cells transfected with miR-21 mimics were significantly upregulated when compared with the other four groups (P<0.01). These findings indicated that miR-21 may target and regulate the expression of the Bcl-2 gene in OCI-LY3 cells.

Discussion

A number of studies have demonstrated that miRNA exhibited differential expression levels in various tumors, and they may interplay with tumor suppressor genes or oncogenes to affect tumorigenesis (18). Indeed, significant overexpression of miR-21 has been reported in glioma (19), pancreatic cancer (20), lung cancer (21), leukemia (22), and lymphoma (23). In the present study, we examined miR-21 expression levels in both DLBCL and normal lymph node tissues. The present results suggest that miR-21 was upregulated in DLBCL tissues and its expression in non-GCB tissues was higher when compared with GCB tissues. These findings were consistent with the results from previous studies (24,25).

Bcl-2 directly participates in cell apoptosis and functions as an anti-apoptotic substance (26); furthermore, it has been indicated that Bcl-2 exhibited low expression levels in apoptotic cells (27). Moreover, Bcl-2 expression is associated with the development of various types of cancer, including breast cancer (28), non-small cell lung cancer (29), nasopharyngeal cancer (30), gastric cancer (31) and non-Hodgkin B-cell lymphoma (32-34). Immunohistochemical results from the present study revealed that the Bcl-2 protein was overexpressed in DLBCL tissues and that Bcl-2 protein expression levels exhibited a significant difference between GCB-DLBCL and non-GCBDLBCL tissues. Therefore, we hypothesized that Bcl-2 was heterogeneously expressed in different histological subtypes.

Additionally, a positive correlation was determined between miR-21 and Bcl-2 expression levels in both GCB-DLBCL and non-GCBDLBCL tissues. The dual-luciferase reporter assay was conducted to illuminate the potential mechanisms. The results indicated that Bcl-2 was a target gene of miR-21 and the Bcl-2 expression levels may be increased through the direct binding of miR-21 to the 3'UTR of Bcl-2 mRNA. However, further comprehensive research is required to confirm these findings.

miR-21 is believed to be a multi-functional miRNA involved in the proliferation, differentiation and anti-apoptosis of cancer cells (35). A previous study has reported that miR-21 was able to increase cell growth and inhibit apoptosis in DLBCL (36). Our findings revealed that upregulation of miR-21 not only exacerbated cell proliferation, but also suppressed the apoptosis of DLBCL cells. However, the inhibition of miR-21 expression may inhibit the proliferation of DLBCL cells and promote the apoptosis of DLBCL cells. Consequently, the present study provides evidence that miR-21 may regulate cell viability and apoptosis in DLBCL and miR-21 mimics may increase Bcl-2 expression levels in DLBCL OCI-LY3 cells. Furthermore, downregulation of miR-21 may contribute to the significant decrease in Bcl-2 expression levels observed in both mRNA and protein levels in OCI-LY3 cells. Our data indicates that the expression of Bcl-2 was modulated by miR-21, which may decrease cell apoptosis through its anti-apoptosis effects via upregulating Bcl-2 gene expression.

Caspase-3 is a member of the caspase protease family and functions as a pivotal participant in cellular apoptosis (37). The activation of caspase-3 has been identified in various types of cells observed with apoptosis (38). Caspase-3 was previously revealed as a downstream molecule of the Bcl-2

family (37). The present study findings were consistent with these previous reports and suggested that higher caspase-3 activity along with increased apoptosis was reflected in cells transfected with Bcl-2 siRNA, whereas lower caspase-3 activity accompanied by decreased apoptosis was observed in cells transfected with miR-21 mimics. Therefore, our data provided evidence that caspase-3 may participate in cellular apoptosis and an underlying interaction may exist between Bcl-2 and caspase-3.

The present study provides evidence that miR-21 may exacerbate the viability of DLBCL cells and inhibit cell apoptosis by positively regulating Bcl-2 expression in DLBCL. Since only one DLBCL cell line (OCI-LY3) was used to detect interactive effects between miR-21 and its target gene, Bcl-2, on tumor proliferation and apoptosis in the present study, the molecular effect of miR-21 and Bcl-2 on tumorigenesis should be further studied.

In conclusion, miR-21 expression was upregulated in DLBCL tissues and the expression of miR-21 was positively correlated with Bcl-2 expression. These findings further demonstrated that miR-21 regulates Bcl-2 expression in a direct manner in DLBCL. Notably, the present study provided evidence that miR-21 may exacerbate the viability of DLBCL cells and inhibit cell apoptosis by targeting Bcl-2. Hence, both Bcl-2 and miR-21 are likely to serve as effective targets for developing alternative treatments for DLBCL.

References

1. Flowers CR, Sinha R and Vose JM: Improving outcomes for patients with diffuse large B-cell lymphoma. CA Cancer J Clin 60: 393-408, 2010.
2. De Roos AJ, Davis S, Colt JS, Blair A, Airola M, Severson RK, Cozen W, Cerhan JR, Hartge P, Nuckols JR and Ward MH: Residential proximity to industrial facilities and risk of non-Hodgkin lymphoma. Environ Res 110: 70-78, 2010.
3. Chang CM, Schroeder JC, Olshan AF, Dunphy CH, Huang WY, Baric RS, Conway K, Cerhan JR, Lynch CF, Rothman N, *et al*: A case-control study of tobacco use and other non-occupational risk factors for lymphoma subtypes defined by t(14; 18) translocations and bcl-2 expression. Cancer Causes Control 21: 1147-1154, 2010.
4. Thompson CA, Habermann TM, Wang AH, Vierkant RA, Folsom AR, Ross JA and Cerhan JR: Antioxidant intake from fruits, vegetables and other sources and risk of non-Hodgkin's lymphoma: The Iowa women's health study. Int J Cancer 126: 992-1003, 2010.
5. Frankenfeld CL, Cerhan JR, Cozen W, Davis S, Schenk M, Morton LM, Hartge P and Ward MH: Dietary flavonoid intake and non-Hodgkin lymphoma risk. Am J Clin Nutr 87: 1439-1445, 2008.
6. Fisher SG and Fisher RI: The epidemiology of non-Hodgkin's lymphoma. Oncogene 23: 6524-6534, 2004.
7. Wang SS, Slager SL, Brennan P, Holly EA, De Sanjose S, Bernstein L, Boffetta P, Cerhan JR, Maynadie M, Spinelli JJ, *et al*: Family history of hematopoietic malignancies and risk of non-Hodgkin lymphoma (NHL): A pooled analysis of 10 211 cases and 11 905 controls from the international lymphoma epidemiology consortium (InterLymph). Blood 109: 3479-3488, 2007.
8. Mazan-Mamczarz K and Gartenhaus RB: Role of microRNA deregulation in the pathogenesis of diffuse large B-cell lymphoma (DLBCL). Leuk Res 37: 1420-1428, 2013.
9. Psathas JN, Doonan PJ, Raman P, Freedman BD, Minn AJ and Thomas-Tikhonenko A: The Myc-miR-17-92 axis amplifies B-cell receptor signaling via inhibition of ITIM proteins: A novel lymphomagenic feed-forward loop. Blood 122: 4220-4229, 2013.
10. Calin GA and Croce CM: MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857-866, 2006.
11. Tagawa H, Ikeda S and Sawada K: Role of microRNA in the pathogenesis of malignant lymphoma. Cancer Sci 104: 801-809, 2013.
12. Jardin F and Figeac M: MicroRNAs in lymphoma, from diagnosis to targeted therapy. Curr Opin Oncol 25: 480-486, 2013.
13. Bai H, Wei J, Deng C, Yang X, Wang C and Xu R: MicroRNA-21 regulates the sensitivity of diffuse large B-cell lymphoma cells to the CHOP chemotherapy regimen. Int J Hematol 97: 223-231, 2013.
14. Iqbal J, Neppalli VT, Wright G, Dave BJ, Horsman DE, Rosenwald A, Lynch J, Hans CP, Weisenburger DD, Greiner TC, *et al*: BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. J Clin Oncol 24: 961-968, 2006.
15. Swerdllow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J and Vardiman JW: WHO classification of tumours of haematopoietic and lymphoid tissues, 4th Edition. Vol 2. IARC, 2008.
16. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Müller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, *et al*: Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103: 275-282, 2004.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
18. Zhang B, Pan X, Cobb GP and Anderson TA: microRNAs as oncogenes and tumor suppressors. Dev Biol 302: 1-12, 2007.
19. Shi L, Chen J, Yang J, Pan T, Zhang S and Wang Z: MiR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity. Brain Res 1352: 255-264, 2010.
20. Giovannetti E, Funel N, Peters GJ, Del Chiaro M, Erozenci LA, Vasile E, Leon LG, Pollina LE, Groen A, Falcone A, *et al*: MicroRNA-21 in pancreatic cancer: Correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. Cancer Res 70: 4528-4538, 2010.
21. Xu LF, Wu ZP, Chen Y, Zhu QS, Hamidi S and Navab R: MicroRNA-21 (miR-21) regulates cellular proliferation, invasion, migration, and apoptosis by targeting PTEN, RECK and Bcl-2 in lung squamous carcinoma, Gejiu City, China. PLoS One 9: e103698, 2014.
22. Medina PP, Nolde M and Slack FJ: OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature 467: 86-90, 2010.
23. Chen L, Pei JH and Chen HM: Effects of continuous positive airway pressure treatment on glycaemic control and insulin sensitivity in patients with obstructive sleep apnoea and type 2 diabetes: A meta-analysis. Arch Med Sci 10: 637-642, 2014.
24. Xiao M, Guo L, Wang T, Zhu T, Jia L, Chen L and Wen F: Interleukin-1B-31T/C promoter polymorphism and chronic obstructive pulmonary disease risk: A meta-analysis. Arch Med Sci 10: 434-438, 2014.
25. Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, Cattan H, Enver T, Mager R, Boultwood J, *et al*: MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. Int J Cancer 121: 1156-1161, 2007.
26. Liu N, Zheng Y, Zhu Y, Xiong S and Chu Y: Selective impairment of CD4+CD25+Foxp3+ regulatory T cells by paclitaxel is explained by Bcl-2/Bax mediated apoptosis. Int Immunopharmacol 11: 212-219, 2011.
27. Yang Q, Yang K and Li A: microRNA-21 protects against ischemia-reperfusion and hypoxia-reperfusion-induced cardiocyte apoptosis via the phosphatase and tensin homolog/Akt-dependent mechanism. Mol Med Rep 9: 2213-2220, 2014.
28. Xiong Y, Ma XY, Zhang Z, Shao ZJ, Zhang YY and Zhou LM: Apoptosis induced by β,β-dimethylacrylshikonin is associated with Bcl-2 and NF-κB in human breast carcinoma MCF-7 cells. Oncol Lett 6: 1789-1793, 2013.
29. Li Y, Zhang S, Geng JX and Hu XY: Curcumin inhibits human non-small cell lung cancer A549 cell proliferation through regulation of Bcl-2/Bax and cytochrome C. Asian Pac J Cancer Prev 14: 4599-4602, 2013.
30. Li Y, Yan L, Zhang W, Wang H, Chen W, Hu N and Ou H: miR-21 inhibitor suppresses proliferation and migration of nasopharyngeal carcinoma cells through down-regulation of BCL2 expression. Int J Clin Exp Pathol 7: 3478-3487, 2014.

31. Wei B, Song Y, Zhang Y and Hu M: microRNA-449a functions as a tumor-suppressor in gastric adenocarcinoma by targeting Bcl-2. Oncol Lett 6: 1713-1718, 2013.
32. Mestre-Escorihuela C, Rubio-Moscardo F, Richter JA, Siebert R, Climent J, Fresquet V, Beltran E, Agirre X, Marugan I, Marín M, *et al*: Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. Blood 109: 271-280, 2007.
33. Song B, Ji W, Guo S, Liu A, Jing W, Shao C, Li G and Jin G: miR-545 inhibited Pancreatic ductal adenocarcinoma growth by targeting RIG-I. FEBS Lett 588: 4375-4381, 2014.
34. Thompson RC, Vardinogiannis I and Gilmore TD: The sensitivity of diffuse large B-cell lymphoma cell lines to histone deacetylase inhibitor-induced apoptosis is modulated by BCL-2 family protein activity. PLoS One 8: e62822, 2013.
35. Pan X, Wang ZX and Wang R: MicroRNA-21: A novel therapeutic target in human cancer. Cancer Biol Ther 10: 1224-1232, 2010.
36. Gu L, Song G, Chen L, Nie Z, He B, Pan Y, Xu Y, Li R, Gao T, Cho WC and Wang S: Inhibition of miR-21 induces biological and behavioral alterations in diffuse large B-cell lymphoma. Acta Haematol 130: 87-94, 2013.
37. Tomek M, Akiyama T and Dass CR: Role of Bcl-2 in tumour cell survival and implications for pharmacotherapy. J Pharm Pharmacol 64: 1695-1702, 2012.
38. Snigdha S, Smith ED, Prieto GA and Cotman CW: Caspase-3 activation as a bifurcation point between plasticity and cell death. Neurosci Bull 28: 14-24, 2012.