Downregulation of BCL11A by siRNA induces apoptosis in B lymphoma cell lines

YANGJUN GAO[1], HONG WU[1], DONGMEI HE[1], XIAOMAO HU[1] and YANGQIU LI[1,2]

[1]Institute of Hematology, Medical College; [2]Key Laboratory for Regenerative Medicine of Ministry of Education, Jinan University, Guangzhou, Guangdong 510632, P.R. China

Abstract. The B-cell chronic lymphocytic leukemia (CLL)/lymphoma 11A gene (BCL11A) encodes a krüppel-like zinc finger protein, which is important in thymopoiesis and has been associated with hematopoietic malignancies. In this study, we investigated whether the downregulation of BCL11A mRNA by small interference RNA (siRNA) was capable of inducing apoptosis, and tested the effect of BCL11A siRNA combined with BCL2 siRNA in B lymphoma cell lines (SUDHL6, EB1). BCL11A siRNA was transfected into SUDHL6, EB1 cells with HiPerfect transfection reagents. After transient transfection with BCL11A siRNA, the expression levels of BCL11A mRNA and protein were assayed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot analysis. The cell proliferation was determined by a cell counting kit-8 (CCK8) assay. Apoptosis was determined by morphological observation and flow cytometric analysis. The results showed that the expression levels of BCL11A mRNA and protein from SUDHL6, EB1 cells transfected with BCL11A siRNA decreased, compared with either the scrambled negative control siRNA group or untransfected cells group ($P<0.05$). Viability of cells transfected with BCL11A siRNA was less compared to cells transfected with control siRNA and untransfected SUDHL6, EB1 cells, respectively ($P<0.05$). BCL11A siRNA induced apoptosis in both SUDHL6 and EB1 cells. BCL11A siRNA combined with BCL2 siRNA significantly inhibited cell growth. Apoptotic rates of SUDHL6, EB1 cells treated with BCL11A siRNA combined with BCL2 siRNA significantly increased ($P<0.05$), compared with either the scrambled control (Sc) siRNA and BCL2 siRNA combination or BCL2 or BCL11A siRNA-treated cells alone. Findings of this study suggest the downregulation of BCL11A mRNA by siRNA was able to induce apoptosis. Moreover, BCL11A siRNA combined with BCL2 siRNA increased apoptosis in SUDHL6, EB1 cells. Thus, suppression of BCL11A expression may be a useful approach in the treatment of B lymphoma.

Introduction

The B-cell chronic lymphocytic leukemia (CLL)/lymphoma 11 (BCL11) gene family members, including the BCL11A and BCL11B genes, encode krüppel-like C_2H_2 zinc finger proteins, which initially have been identified as transcriptional repressors (1-3). The BCL11A gene was initially found in mice as an ecotropic retrovirus integration site (Evi9), also known as chicken ovalbumin upstream promoter transcription factor-interacting protein 1 (CTIP1) (1). BCL11A was previously shown to be associated with lymphocyte proliferation and differentiation (2,4). Abnormalities involving BCL11A have been detected in a variety of B-cell malignancies and certain subsets of acute myeloid leukemia in humans (3,5-6). We and others reported high levels of BCL11A mRNA expression in the majority of B-CLL, B-lymphoma and B-cell leukemia/lymphoma cell lines (3,7-10). However, the precise function of this transcription factor in B-cell malignancies still remains poorly characterized. Little is known whether the BCL11A gene is associated with cell apoptosis. Double-stranded RNA-dependent post-transcriptional gene silencing, also known as RNA interference (RNAi), is a phenomenon where double-stranded small interfering RNA (siRNA) complexes are able to target specific genes. In the present study, we firstly report that the suppression of BCL11A by RNAi is capable of inducing apoptosis in the diffuse large B-cell lymphoma (SUDHL6) and Burkitt's lymphoma (EB1) cell lines.

BCL2, a member of the BCL2 family, plays an important role in programmed cell death/apotosis (11). It has been shown that siRNA targeting against BCL2 mRNA was able to effectively induce apoptosis of lymphoma and leukemia cells (11-13). However, whether BCL11A siRNA combined with BCL2 siRNA enhanced apoptosis of lymphoma SUDHL6, EB1 cells remains uncertain. Therefore, we also investigated whether BCL11A siRNA combined with BCL2 siRNA was able to enhance cell apoptosis. The findings indicated that the downregulation of BCL11A serves as a new therapeutic option in B-cell malignancies.

Correspondence to: Dr Dongmei He, Institute of Hematology, Medical College, Jinan University, No. 601 West Huangpudadao, Guangzhou, Guangdong 510632, P.R. China
E-mail: thedm@jnu.edu.cn

Materials and methods

Reagents. BCL11A-specific (BCL11A-585,2292) siRNAs, BCL2-specific (BCL2-195) siRNA and the corresponding non-silencing scrambled negative control (Sc) siRNA were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). RPMI-1640 and newborn calf serum were purchased from Gibco (Carlsbad, CA, USA).

Cell culture and transfection with siRNA. The SUDHL6, EB1 cell lines were kindly provided by Professor Ailin Guo from the Department of Plant Pathology (Cornell University, Ithaca, NY, USA). The cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified incubator at 5% CO_2. SUDHL6, EB1 cells in the exponential phase of growth were grown for 24 h, and transfected with BCL11A siRNA (si585, si2292) using HiPerfect (Qiagen, Valencia, CA, USA), following the manufacturer's protocols. Mock control cells (mock) and sc siRNA were transfected with HiPerfect agents. The total concentration of siRNA applied in each case was kept constant at 100 nM. For treatments with combined BCL11A siRNA and BCL2 siRNA, the ratio of BCL11A siRNA to BCL2 siRNA was kept at 1:1. At 48 and 72 h of transfection, silencing of BCL11A gene was examined.

Assay of BCL11A mRNA by qRT-PCR. Total RNA was isolated by TRIzol® total RNA isolation system (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed into the first-strand complementary DNA (cDNA) using random hexamer primers and the reverse transcriptase Superscript II® kit (Invitrogen Life Technologies), according to the manufacturer's instructions. RNA purity and concentration was measured with a spectrophotometer. The $2^{-\Delta Ct}$ x100% method [ΔCt=CT(BCL11A) - CT(GAPDH)] was used to analyze the relative changes in gene expression from qRT-PCR experiments with SYBR-Green I (14,15). The primers were synthesized (Shanghai Genechem Biotechnology Co., Ltd., China) as previously described (16,17). The primer sequences used in the PCR reactions were: BCL11A sense: 5'-AACCCCA GCACTTAAGCAAA-3' and antisense: 5'-GGAGGTCATGA TCCCCTTCT-3'; GAPDH sense: 5'-ACCCAGAAGACTGTG GATGG-3' and antisense: 5'-TTCAGCTCAGGGATGAC CTT-3'. The total reaction volume was 20 μl. Reaction conditions were initiated with the enzyme activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec and 80°C for 5 sec. At the end of each run a melting curve was performed starting at a temperature of 65°C and rising up to 95°C with an increase of 1°C per 2 sec to verify primer specificities. Each run was completed with a melting curve analysis to confirm the specificity of the amplification and the absence of primer dimmers. The qRT-PCR was repeated in at least three separate experiments.

Western blot analysis. Cells were washed with phosphate-buffered saline (PBS) (10 mM, pH 7.4), incubated in 200 ml of cell lysis buffer (50 mmol/l Tris-HCl, 5 mmol/l EDTA, 25% sucrose, 0.6 ml of 100 mg/ml phenylmethylsulphonyl fluoride, 2.4 ml 2-mexca-ptoethanol) on ice for 30 min, and centrifuged at 13,000 x g for 45 min at 4°C. The protein of cell lysate was determined by a Bio-Rad Protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Whole cell extracts equivalent to 100 μg of total protein were separated by 8% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Gibco-BRL, Carlsbad, CA, USA). The blot was placed in blocking buffer (10% non-fat dry milk, 1% Tween-20; in 20 mM Tris-buffered saline, pH 7.5) for 1 h at room temperature and incubated with appropriate anti-human primary antibody (Abcam, Cambridge, MA, USA; mouse anti-human BCL11A/IgG, 1:1,000; mouse anti-human GAPDH/IgG, 1:2000) overnight at 4°C. Blots were incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3,000) for 1 h and detected by chemiluminescence using ECL Hyperfilm.

Assay of cell viability. For the quantitative determination of cellular proliferative activity, the cell counting kit-8 (CCK8; Dojindo, Tokyo, Japan) assay was carried out following transfection of SUDHL6 and EB1 cells with BCL11A siRNA alone or in combination with BCL2 siRNA at 24, 48 and 72 h. The cells were washed, counted and seeded at a density of $4x10^5$/ml cells per well in 96-well plates. Six hours later, BCL11A siRNA alone or in combination with BCL2 siRNA was added to the cells. At 24, 48 and 72 h after transfection, CCK8 solution was added 4 h before the end of incubation. Cell viability was measured with a spectrophotometer at an absorbance of 450 nm. The inhibition rates of cell growth were calculated according to the formula: inhibition rate (%) = (1-mean absorbance of treatment group/mean absorbance of untreatment group) x100%.

Cell apoptosis assay. The transfected SUDHL6, EB1 cells were harvested after treatment. Morphology was determined with Hoechst 33258 (Invitrogen) following incubation for 72 h. Cells were washed with PBS three times and then stained with 10 μl Hoechst 33342 nuclear dye for 10 min at 37°C. Cells were again washed three times with PBS and images were obtained by fluorescence microscopy (Leica, Wetzlar, Germany). Apoptosis detection kit (Annexin V BioVision, Palo Alto, CA, USA) was performed as per the manufacturer's instructions. Briefly, cells were centrifuged, washed with cold PBS, and resuspended in 500 μl binding buffer. Fluorescein isothiocyanate-conjugated Annexin V (10 μl) and propidium iodide (PI) (10 μl) were added to each sample, and the mixture was incubated at 4°C in the dark for 5 min. The cells were immediately subjected to fluorescence-activated cell sorting (FACS) analysis (BD FACSCalibur; BD Biosciences, San Jose, CA, USA). The percentage of the early and late apoptotic cells in each group was determined.

Statistical analysis. Data were shown as the mean ± standard deviation (SD). Statistical comparisons were made by analysis of variance (ANOVA). Differences were deemed significant at α=0.05. Statistical analysis was carried out using SPSS 13.0.

Results

BCL11A siRNAs suppressed BCL11A expression at the mRNA and protein levels in SUDHL6 and EB1 cells. To determine the

Table I. Effect of BCL11A siRNA on BCL11A mRNA expression levels by qRT-PCR at 48 h after transfection in SUDHL6 and EB1 cells (%, mean ± SD, n=3).

Cell lines	si585	si2292	Sc	Mock	Cells
SUDHL6	36.91±2.63[a]	37.73±1.97[a]	62.11±2.57	63.43±2.69	66.32±3.24
EB1	32.09±2.08[a]	38.69±3.62[a]	51.46±3.37	53.86±1.93	55.93±3.37

[a]$P<0.05$; SD, standard deviation; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Sc, scrambled control.

Table II. Inhibition rates of SUDHL6 and EB1 cell growth by CCK8 assay after transfection with BCL11A siRNA (%, ± SD, n=3).

Cell lines	si585	si2292	Mock	Sc
SUDHL6 (h)				
24	9.66±1.65[a]	12.96±1.16[a]	3.39±0.79	4.43±0.85
48	39.70±2.95[a]	36.19±1.34[a]	3.23±1.53	9.63±2.84
72	44.90±2.47[a]	42.27±4.10[*]	3.23±0.86	9.39±2.41
EB1 (h)				
24	35.54±2.17[a]	15.21±2.09[a]	5.91±0.27	3.52±1.17
48	37.91±3.34[a]	33.48±2.75[a]	3.85±0.69	8.01±2.60
72	44.38±6.21[a]	38.42±3.67[a]	3.54±1.21	2.37±0.87

[a]$P<0.05$; CCK8, cell counting kit-8; SD, standard deviation; Sc, scrambled control.
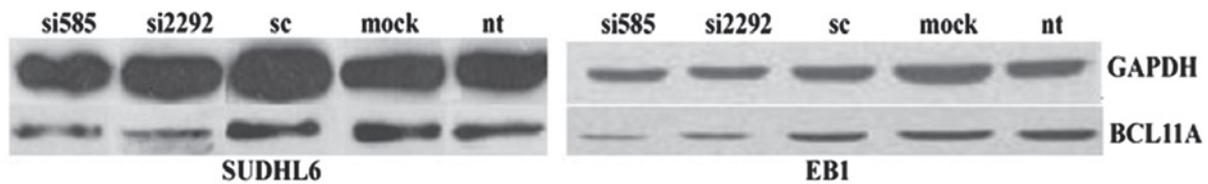
Figure 1. Effect of BCL11A siRNA on BCL11A protein expression levels in SUDHL6 and EB1 cells. BCL11A protein levels were determined using western blot analysis at 72 h after transfection with BCL11 siRNA585 (si585) or BCL11A siRNA2292 (si2292) is shown. Non-silencing scrambled siRNA (Sc)-treated, mock-transfected (mock) and non-treated (nt) cells were used as controls. GAPDH served as a loading control.

efficiency of BCL11A inhibition following siRNA treatment, BCL11A expression was analyzed at the mRNA and protein level. The qRT-PCR data shown include at least three independent experiments. According to the relative qRT-PCR formula: $2^{-\Delta Ct}$ x100% (14,15) the relative expression levels of BCL11A mRNA in SUDHL6 and EB1 cells treated with BCL11A siRNA (siRNA585 and 2292) were significantly lower than cells treated with the control siRNA (P<0.05), at 48 h after transient transfection (Table I). By contrast, the control siRNA had no effect on the expression of BCL11A mRNA.

BCL11A protein levels were assayed by western blot analysis. Western blot analysis confirmed the results of the qRT-PCR analysis (Fig. 1). Expression of BCL11A protein significantly decreased in the two cell lines treated with BCL11A siRNA as compared with either the negative control siRNA group or untreated cells group. No difference in BCL11A levels among the control siRNA group, mock-transfected-treated cells and

untransfected cells was found. The two BCL11A siRNAs were equally effective at reducing BCL11A mRNA and protein. No statistically significant difference was detected between the two cancer cell lines.

The effects of BCL11A siRNA on cell proliferation in SUDHL6 and EB1 cells. For the quantitative determination of proliferative activity, a CCK8 assay was carried out. A summary of experiments measuring cell proliferation at 24, 48 and 72 h is shown in Table II. The growth of SUDHL6 and EB1 cells was inhibited in a time-dependent manner at a concentration of 100 nM siRNA (siRNA585 and 2292) 24, 48 and 72 h after transfection. Of note, a difference was detected in cell viability between siRNA585 and 2292 at 24 h in EB1 cells. However, no statistically significant difference was found between siRNA585 and 2292 at 48 and 72 h, nor between the two cell lines.

Table III. Rate of apoptosis by Annexin V analysis at 72 h after transfection with BCL11A siRNA in SUDHL6 and EB1 cells (%, mean ± SD, n=3).

Cell lines	si585	si2292	Sc	Mock	Cells
SUDHL6	32.00±3.15[a]	26.53±2.94[a]	5.12±3.18	2.90±2.37	2.11±1.39
EB1	29.43±2.38[a]	23.90±2.76[a]	4.66±2.12	3.24±1.17	1.76±0.48

[a]P<0.05; SD, standard deviation; Sc, scrambled control.
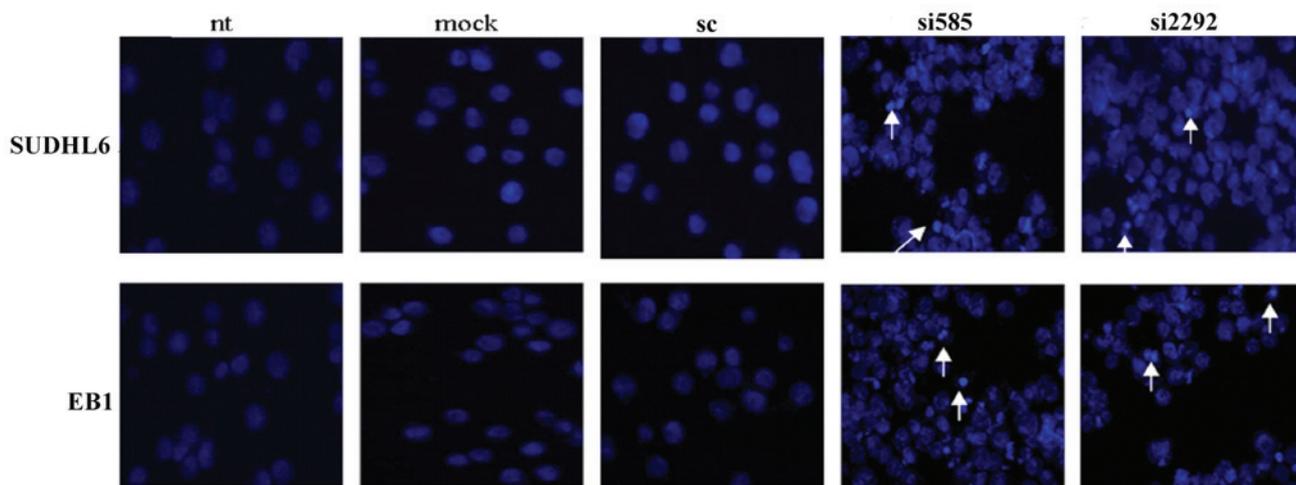
Figure 2. The morphology effect of BCL11A siRNA on apoptosis of SUDHL6 and EB1 cells. Nuclear morphology of cells stained with Hoechst 33258 was analyzed by fluorescence microscopy (magnification, x200) at 72 h after transfection with BCL11A siRNA (si585, si2292). Non-silencing scrambled siRNA (Sc), mock-transfected (mock) and untreated (nt) cells were used as controls. Data are representative microscopic images.

Effects of BCL11A siRNA on cell apoptosis in SUDHL6 and EB1 cells. Two methods were used to assess apoptosis: i) Hoechst staining and ii) Annexin V-FITC/PI double staining by FACS analysis. SUDHL6, EB1 cells exhibited characteristic of apoptosis. As shown in Fig. 2, in the control groups, the cells demonstrated normal nuclear morphology, while at 72 h after transfection with BCL11A siRNA in SUDHL6, EB1 cells, a significant nuclear condensation and fragmentation were observed. Annexin V-FITC and PI double staining with FACS were employed to detect the effect of BCL11A siRNA on cell apoptosis. The percentage of Annexin V-FITC/PI-stained cells for early and late apoptotic cells increased, reaching 32.00 and 29.43% for siRNA585 and 26.53 and 23.90 % for siRNA2292 at 72 h in the SUDHL6 and EB1 cell lines, respectively (Table III). Control siRNA-treated, -untreated or mock-transfected cells showed ~1-5% apoptotic cells. No statistically significant difference was detected in cell apoptosis between siRNA585 and 2292, nor between the two cell lines.

Effects of BCL11A siRNA in combination with BCL2 siRNA on cell growth and apoptosis in SUDHL6 and EB1 cells. To investigate the effects of BCL11A siRNA combined with BCL2 siRNA on SUDHL6 and EB1 cells, a CCK8 assay was applied. A summary of experiments measuring cell proliferation at 24, 48 and 72 h is shown in Table IV. Compared to treatments with either BCL11A siRNA or BCL2 siRNA alone, cell viability was effectively reduced by combining BCL11A siRNA and BCL2 siRNA in a time-dependent manner. No difference in cell viability was detected between siRNA585 combined with BCL2 siRNA and siRNA2292 combined with BCL2 siRNA. A large increase was found in AnnexinV-FITC/PI double-positive cells at 72 h after the combined treatment with BCL11A siRNA and BCL2 siRNA in SUDHL6, EB1 cells (Table V). The difference in apoptotic rates was significant, compared to BCL11A siRNA or BCL2 siRNA alone, respectively (P<0.05). No significant difference in apoptosis occurred between those cells treated with siRNA585 combined with BCL2 siRNA and siRNA2292 combined with BCL2 siRNA (P>0.05).

Discussion

The BCL11A gene encodes a C_2H_2 zinc finger protein, which is a krüppel transcription factor and is closely associated with B cell proliferation and differentiation (2,4). Alternative splicing within the BCL11A locus leads to the creation of four major protein isoforms: extra long (XL), long (L), short (S), and extra short (XS) (3,7). The BCL11A-XL mRNA is the largest and most abundant transcript (3). Our previous results showed the expression level of BCL11A in the B-cell acute lymphocyte leukemia patients and B lymphoma cell lines (including SUDHL6 and EB1) to be increased significantly, when compared to the T-cell acute lymphocyte leukemia and

Table IV. Inhibition rates of SUDHL6 and EB1 cell growth by CCK8 assay following transfection with BCL11A siRNA in combination with BCL2 siRNA (%, ± SD, n=3).

Cell lines	si585	si2292	siBCL2	si585+siBCL2	si2292+siBCL2
SUDHL6 (h)					
24	9.66±1.65	12.96±1.16	14.2±2.25	53.42±4.87[a]	48.10±3.90[a]
48	39.70±2.95	36.19±1.34	40.16±2.17	66.58±3.96[a]	62.39±3.42[a]
72	44.90±2.47	42.27±4.10	47.58±3.66	70.14±5.19[a]	65.48±3.29[a]
EB1(h)					
24	35.54±2.17	15.21±2.09	37.43±4.30	55.90±2.90[a]	53.12±4.01[a]
48	37.91±3.34	33.48±2.75	37.41±4.21	59.97±6.30[a]	56.48±5.92[a]
72	44.38±6.21	38.42±3.67	42.84±3.09	65.24±3.98[a]	61.11±4.78[a]

[a]P<0.05; CCK8, cell counting kit-8; SD, standard deviation; siBCL2, BCL2 siRNA.

TableV. Rate of apoptosis by Annexin V analysis at 72 h after trancfection with BCL11A siRNA in combination with BCL2 siRNA in SUDHL6 and EB1 cells (%, ± SD, n=3).

Cell lines	si585	si2292	siBCL2	si585+siBCL2	si2292+siBCL2
SUDHL6	32.00±3.15	26.53±2.94	25.27±1.49	55.17±3.58[a]	41.05±6.67[a]
EB1	29.43±2.38	23.90±2.76	18.29±2.39	44.13±5.71[a]	38.72±7.83[a]

[a]P<0.05; SD, standard deviation; siBCL2, BCL2 siRNA.

the healthy control groups (9,10). However, whether suppression of BCL11A was likely to induce apoptosis of cells has yet to be reported.

To clarify the role of BCL11A in B lymphoma cell proliferation and apoptosis, RNA interference was employed to inhibit the expression of BCL11A in two different human B-cell lymphoma-derived cell lines. Our results have shown that the expression levels of BCL11A mRNA and protein were decreased, following transfection of either SUDHL6 or EB1 cells with BCL11A siRNA. Two different siRNA targeting different regions (BCL11A-585, 2292) of the BCL11A sequence yielded congruent results. Viability of cells transfected with either BCL11A siRNA585 or 2292 showed a decrease in both SUDHL6 and EB1 cells. Moreover, findings of this study have also shown that the downregulation of BCL11A expression by siRNA is able to induce apoptosis in both SUDHL6 and EB1 cells. Taken together, the results suggest that BCL11A siRNA-mediated growth suppression of SUDHL6, EB1 cells occurs via the induction of apoptosis.

Our findings are in accordance with the known antiapoptotic role of BCL11B in T-cell malignancies (18-20). Recently, it has been shown that BCL11B is one of the necessary genes for T-cell differentiation and is associated with T-cell malignancies (21-23). BCL11B was 67% identical to BCL11A at the nucleotide level and 61% identical overall at the protein level. Similar to BCL11A, BCL11B is the homologue of mouse CTIP-2 and is 86% identical at the protein level, which contains 6 C_2H_2 zinc fingers and proline-rich and acidic regions with 95% identity in the zinc finger domains (3,24). Therefore, we

suggest that BCLl11A and BCL11B has similar antiapoptotic action in malignant lymphocyte cells. However, the underlying molecular mechanisms by which BCL11A induces apoptosis remain to be elucidated. Taken together, our results have demonstrated that BCL11A may have an antiapoptotic role in malignant B lymphocyte cell lines. Our results therefore provide evidence for a major role of BCL11A in promoting the survival of malignant B cells.

BCL2 is known to be a prominent member of the BCL2 family which regulates the induction of apoptotic cell death by a wide variety of stimuli. Previous studies have shown that a reduction in BCL2 expression by siRNA is capable of inducing apoptosis in Raji cells (12). In the present study, as expected, BCL11A siRNA treatment in combination with BCL2 siRNA decreased cell proliferation and increased the number of apoptotic cells compared with BCL11A or BCL2 siRNA- transfected cells alone. Both these synergistic apoptotic effects and cell number reduction in the combination treatment are statistically significant. It has been shown that the blockage of multiple physiological events mediating malignancy and tumor cell survival, or blockage of a single critical path at multiple points to maximize inhibition of that path, is likely to result in greater-than-additive inhibition of tumor cell growth and viability (25). Similarly, in more recent studies, *in vitro* RNAi experiments conducted against hepatitis C virus using combinatorial treatment with two siRNAs elicited a significant increase in antiviral effects (26).

Our data indicate that the downregulation of BCL11A by siRNA induced apoptosis in SUDHL6, EB1 cells. BCL11A

siRNA may therefore be useful for the treatment of tumors exhibiting an overexpression of BCL11A. Findings of the present study suggest that the selective blockade of BCL11A triggers apoptosis and suppresses the transduction of survival signals, leading to cancer cell growth inhibition and enhancing anti-tumor activity. Additionally, the combination of BCL11A and BCL2 siRNA produced a greater inhibitory effect on the cell proliferation of SUDHL6, EB1 cells compared to the two treatments given separately, indicating that BCL11A knockdown caused a marked synergistic enhancement of BCL2 siRNA-induced apoptosis. Our data therefore suggests that the downregulation of BCL11A expression is a relevant therapeutic strategy in B-cell lymphoma.

Acknowledgements

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