Growth inhibition and apoptosis of human B-cell lymphoma *in vitro* and *in vivo* by Bcl-2 short hairpin RNA

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Abstract. Bcl-2 is overexpressed in various types of human tumors, including Burkitt's lymphoma, and it is involved in tumorigenesis and chemoresistance, therefore, it is regarded as a potential target of gene therapy. In this study, RNA interference using short hairpin RNA (shRNA)-mediated RNA interference was introduced into Burkitt's lymphoma Raji cells to validate its effects on Bcl-2 expression and cell proliferation *in vitro* and *in vivo*. We constructed two types of Bcl-2 shRNA plasmid (pGenesil-1-Bcl-2-1 and pGenesil-1-Bcl-2-2) and negative control shRNA plasmid (pGenesil-1-NC) and stably transfected them into Raji cells. The expression levels of Bcl-2 mRNA and protein were assayed by RT-PCR, flow cytometry and western blotting. Cell proliferation was determined by cell count assay. The antitumor activities and apoptosis of the two types of Bcl-2 shRNA plasmid were evaluated in BALB/c nude mice bearing Burkitt's lymphoma inoculated with Raji cells. The results showed that the expression levels of Bcl-2 mRNA and protein decreased, compared with either the pGenecil-1-NC or the untransfected cell group ($P<0.05$). The cell proliferation assay showed that Bcl-2 shRNA significantly inhibited the growth of Raji cells ($P<0.01$). Furthermore, the tumor growth of the Bcl-2 shRNA cell group was dramatically lower and smaller than that of the negative control or untransfected cell group ($P<0.01$). Bcl-2 protein expression in the untransfected and the pGenesil-1-NC group were markedly higher than that of the pGenesil-1-Bcl-2-1 and the pGenesil-1-Bcl-2 group by immunohistochemistry (both $P<0.01$) and the results using transmission electron microscopy showed that Bcl-2 shRNA significantly induced Raji cell apoptosis. Additionally, the inhibition effect of pGenesil-1-Bcl-2-1 was better than that of pGenesil-1-Bcl-2-2. It has been suggested that vector-based Bcl-2 shRNA could effectively reduce the expression of Bcl-2 and induce apoptosis and growth inhibition of Burkitt's lymphoma Raji cells. Vector-based Bcl-2 shRNA could be a potential gene therapeutic strategy against human Burkitt's lymphoma.

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Introduction

Cancer cells escape apoptosis by a number of mechanisms, among which overexpression of anti-apoptotic genes, such as some members of the Bcl-2 gene family, the IAP family and the Mcl-1 family, have been shown to play a critical role (1-3). The proto-oncogene Bcl-2, discovered in Burkitt's lymphoma, is a prominent member of the Bcl-2 family which prevents apoptosis in various malignancies including Burkitt's lymphoma. Consequently, targeting expression of Bcl-2 has a potential value in Burkitt's lymphoma therapy, and has led to the development of therapeutic strategies to selectively inhibit targeted gene expression. Previous studies have demonstrated that the inhibition of Bcl-2 expression by antisense oligonucleotides (4,5) reduces the growth of Burkitt's lymphoma cells. However, their efficiency is unsatisfactory due to nuclease degradation. Recently, the successful use of small interfering RNAs (siRNAs) in down-regulating gene expression in several model systems led to many attempts to explore this methodology in potentially therapeutic settings. siRNA involves post-transcriptional gene silencing via a process in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence-dependent manner through degradation of the corresponding mRNA. It has been verified as a powerful tool to knock down the expression of a target gene in mammalian cells (6-8). At present, siRNA can be synthesized by chemical synthesis and *in vitro* transcription. The limitations of the two methods are high cost and low stability. Stable gene repression can be achieved in mammalian cells by using vectors to express a small hairpin RNA (shRNA) structure with a U6 or H1 promoter under the direction of RNA polymerase III (9,10).

In this study, a U6 promoter-based vector was used to express shRNA targeting Bcl-2 in Burkitt's lymphoma Raji cells. We investigated whether this technique could be used for the specific inhibition of Bcl-2 overexpression and tested whether this inhibition could result in antitumor effects.

Materials and methods

Cell culture. The human B-cell lymphoma cell line Raji used in this study was maintained in our laboratory. The cells were grown in a suspension of RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China), 5 mmol/l HEPES, 100 U/ml penicillin and 100 U/ml streptomycin in 5% atmospheric CO_2 at 37˚C. The cells were passaged every three days, checked routinely, and found to be free of contamination. When the cells grew to exponential phase, they were harvested and used for *in vitro* and *in vivo* studies.

shRNA design and construction of recombinant plasmid expressing Bcl-2-shRNA. According to the shRNA design principle (11), two 19 bp sequences in Bcl-2 cDNA from GenBank accession number NM-000633.2 as our target sites were designed by using siRNA design software downloaded from the internet (http://www.ambion.com). One was 5'-GTACATCCATTATAAGCTG-3', which corresponds to the nucleotides 544-562, and the other was 5'-CATCGCCCT GTGGATGACT-3', which corresponds to the nucleotides 1009-1027. The secreted sequences were submitted to BLAST search to ensure the only secreted gene was targeted. The negative control scrambled sequence was 5'-GACTTCATAAGG CGCATGC-3', which has no significant homology to mouse or human gene sequences. The oligonucleotides contained a sense strand of 19 nucleotides followed by loop sequence TTC AAGAGA, an antisense strand, a transcription terminator TTTTT, an identification restriction enzyme *Eco*RI site, as well as terminal *Bam*HI and *Hin*dIII restriction enzyme sites (Table I). Double complementary shRNA DNA segments were gained through annealing, named Bcl-2-1 shRNA, Bcl-2-2 shRNA and NC shRNA and then inserted into the *Bam*HI and *Hin*dIII sites of plasmid pGenesil-1 vector, respectively. The recombinant plasmids were evaluated by restriction enzyme cutting and sequencing, and were then designated as pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2 and pGenesil-1-NC, respectively.

Transfection with the shRNA expression vector. For transfection, Raji cells in exponential phase of growth were harvested and washed three times with Opti-MEM (Invitrogen, USA) to replace the culture medium and then $5x10^5$ cells were seeded into wells of a 6-well plate and divided into five groups: group 1, normal cultured Raji cells; group 2, Raji cells transfected with pGenesil-1-Bcl-2-1; group 3, Raji cells transfected with pGenesil-1-Bcl-2-2; group 4, Raji cells transfected with negative control plasmid vector pGenesil-1-NC. Transfection was performed according to the manufacturer's protocols. The ratio of plasmid to Lipofectamine™ 2000 (Invitrogen, USA) was 1:2. Five hours after the transfection, the medium was replaced by the common complete medium again. After 48 h of transfection, cells stably expressing shRNA were established by selection with medium first containing 600 μg/ml G418. The medium was renewed every 3 days. After 15 days selection, the resistant colonies were combined in pools in selective medium. Then, the resistant colonies were further selected by a huge dose G418 (2,000 μg/ml) for 10 days in order to exclude the possibility of non-transfected but G418-resistant colonies,

Table I. DNA sequences of insertion fragments for shRNAs.

Name	Sequences (5'-*Bam*HI+sense+loop+antisense+termination signal+*Eco*RI+*Hin*dIII-3')	Target site
Bcl-2-1 shRNA	5'-*GATTC*GTACATCCATTATAAGCTG**TTCAAGACG**CAGCTTATAATGGATGTACTTTTTTGAATTC*A*-3' 3'-*GC*ATGTAGGTAATATTCGAC**AAGTTCTGC**GTCGAATATTACCTACATGAAAAAACTTAAG*TTCGA*-5'	544-562
Bcl-2-2 shRNA	5'-*GATTC*GCATCGCCCTGTGGATGACT**TTCAAGACG**AGTCATCCACAGGGCGATGTTTTTTGAATTC*A*-3' 3'-*GCGTAGCGGGACACCTACTGA**AAGTTCTGC**TCAGTAGGTGTCCCGCTACAAAAAACTTAAG*TTCGA*-5'	1009-1027
NC shRNA	5'-*GATTC*GACTTCATAAGGCGCATGC**TTCAAGACG**GCATGCGCCTTATGAAGTCTTTTTTGAATTC*A*-3' 3'-*GC*TGAAGTATTCCGCGTACG**AAGTTCTGC**CGTACGCGGAATACTTCAGAAAAAAACTTAAG*TTCGA*-5'	

Restriction enzyme *Eco*RI site is underlined, terminal *Bam*HI and *Hin*dIII restriction enzyme sites are indicated in italics and loop is indicated in bold.

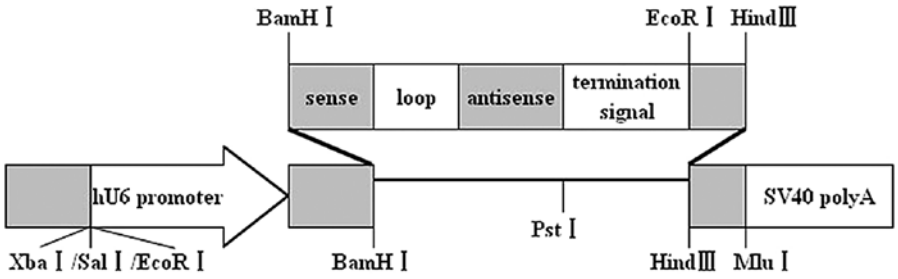

Figure 1. Structure of plasmid pGenesil-1 vector. The shRNA encoding template was inserted between the *Hin*dIII and *Bam*HI restriction sites, and the *Pst*I restriction site of plasmid pGenesil-1 was replaced.

after the huge dose selection of G418, the colonies stably transfected with G418-resistance were amplified and analyzed by RT-PCR, western blot analysis and flow cytometric assays, for subcutaneous tumorigenesis assay in nude mice.

Bcl-2 mRNA expression detected by RT-PCR. Stably transfected and untreated cells were collected and washed with phosphate-buffered saline (PBS). Total cellular RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and concentration were determined by measuring the absorbance (A) at 260 and 280 nm (A_{260}/A_{280}). To generate first-strand cDNA, an oligo$(dT)_{18}$ was used as primer, and 2 μg RNA was reverse-transcribed based on the MMLV First Strand cDNA Synthesis kit (Fermentas, USA) protocols. Subsequently, aliquots of 5 μl cDNA were amplified in a total volume of 25 μl using the polymerase chain reaction (PCR) kit (Fermentas, USA). The sense and antisense primers for Bcl-2 were: 5'-CGCGACT CCTGATTCATT-3', 5'-TGCATTCTTGGACGAGGG-3' (316 bp); the sense and antisense primers for the housekeeping gene β-actin used as an internal control were: 5'-GGACCTGA CTGACTACCTC-3', 5'-TCATACTCCTGCTTGCTG-3' (420 bp), respectively. The cycling conditions were 94˚C for 5 min, followed by 30 cycles of 94˚C for 1 min, 51˚C for 30 sec, and 72˚C for 1 min and a final extension of 72˚C for 10 min. PCR products were separated in 1.5% agarose gels, stained with ethidium bromide and visualized by UV absorption. Densitometric scanning of the bands was performed and relative amount of Bcl-2 mRNA expression was estimated by normalization to the β-actin mRNA detected in the same sample.

Western blot analysis. Cytoplasmic proteins of the groups above were extracted using cytoplasmic extraction reagents (Beyotime Biotechnology, China). Cell lysates were centrifuged at 15,000 rpm for 5 min at 4˚C. Protein content in the supernatants was determined by a BCA protein assay kit. Equal amounts of lysate protein were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membrane was blocked in 5% skimmed milk in TBST buffer at room temperature for 1-2 h with gentle shaking and then incubated overnight at 4˚ with mouse anti-human Bcl-2 mAb (1:1,000) or mouse anti-human β-actin IgG mAb (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG antibody (1:5,000, Immunotech, France). Bands were developed by using ECL substrate and exposed to X-ray film. The expression levels of Bcl-2 and β-actin protein were quantified by densitometry. The signal strength of each Bcl-2 signal was normalized against the corresponding β-actin control.

Bcl-2 protein expression detected by flow cytometry. Transfected cells, as well as untreated cells, were collected and washed twice with PBS and then fixed in 1% paraformaldehyde for 5 min and permeabilized with 0.1% Triton X-100 for 5 min. After two additional rinses with PBS, the cells were incubated with primary mouse anti-human Bcl-2 IgG mAb (Immunotech) for 30 min, followed by FITC-conjugated goat anti-mouse IgG mAb (Immunotech) for 30 min at room temperature in the dark. The cells were then rinsed twice with PBS containing 2% FBS and analyzed by flow cytometry (FCM). Controls consisted of incubation with no primary antibody or incubation but with only the secondary antibody. The experiments were performed in triplicate and the results are given as the means ± SD.

Cell proliferation assay. To determine whether Bcl-2 shRNA could inhibit Raji cell proliferation, Raji cells stably transfected with pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2, pGenesil-NC and untreated cells were plated in 96-well plates at a density of $1x10^4$ cells/well and incubated for 9 days in 5% atmospheric CO_2 at 37˚C. Cells were stained with trypan blue and counted using a hemocytometer. Cell numbers of the above groups were detected on days 1-9. Each experimental condition was performed three times and all data presented as the means ± SD for each group were determined to compose the growth curve.

Treatment in vivo. We then investigated whether Bcl-2 shRNA would alter the tumorigenicity or not. Four-week-old male BALB/c nude mice were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China) and our animal studies were carried out in accordance with established institutional guidelines and approved protocols. An equal number ($3x10^7$ cells in 0.1 ml PBS) of Raji cells stably transfected with pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2, pGenesil-NC control, and untreated cells were harvested, washed with PBS and injected into male 4-week-old BALB/c nude mice (six mice for each group) subcutaneously. The mice were kept in a pathogen-free environment. Tumor sizes were measured every four days

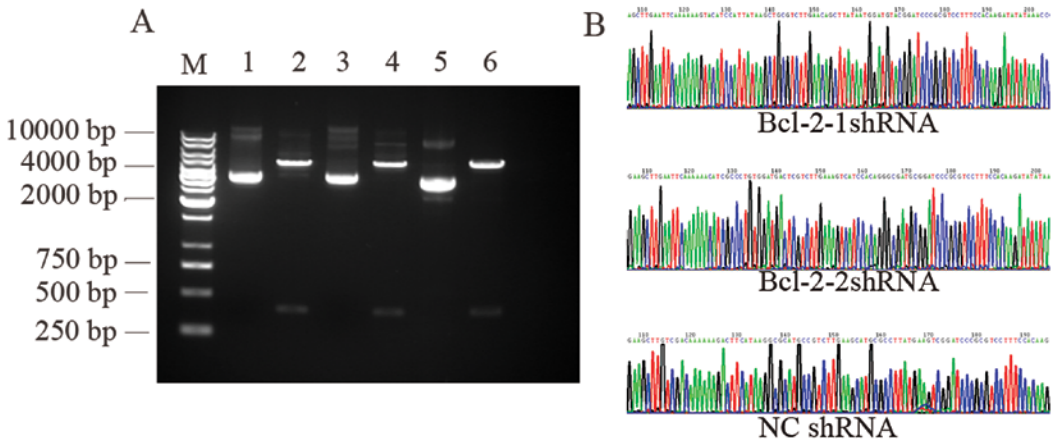

Figure 2. (A) Identification of recombinant plasmids by restriction enzyme digestion. M: 1 kb DNA marker; 1, 3, 5: pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2 and pGenesil-1-NC digested by *Pst*I, respectively. Since the restriction site of enzyme *Pst*I in plasmid pGenesil-1 was replaced by the inserted shRNA segment, the reconstructed plasmids could not be digested by *Pst*I; 2, 4, 6: pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2, pGenesil-1-NC digested by *Eco*RI, respectively. An *Eco*RI enzyme site was designed in each inserted shRNA segment, and the pGenesil-1 plasmid itself carried an *Eco*RI enzyme digestion site. A band approximately 400 bp was cut off by *Eco*RI for pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2 and pGenesil-1-NC, respectively. (B) Recombinant plasmids identified by DNA sequence analysis (the insertion segment).

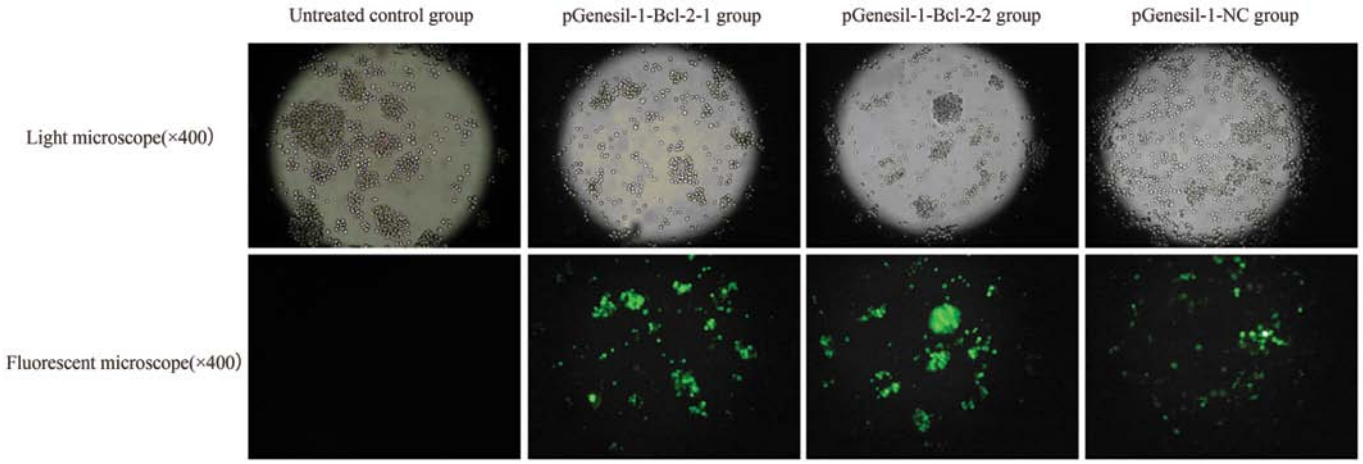

Figure 3. Light and fluorescent images of Raji cells stably transfected with recombinant plasmids (original magnification, x400). The transfected cells were observed as green color under fluorescence microscope in pGenesil-1-Bcl-2-1 cells, pGenesil-1-Bcl-2-2 cells and pGenesil-1-NC cells, while EGFP was not expressed in Raji cells.

and calculated by the following formula: volume (mm^3) = 1/2 $(width)^2$ x length. Forty days after injection, the mice were sacrificed and the weights of the tumors were recorded. The tumors were removed, fixed by 4% polyformaldehyde, paraffin embedded and sectioned for immunohistochemical analysis and transmission electron microscopic examination.

Immunohistochemistry. For the immunohistochemistry, 5 μm-thick paraffin-embedded sample tissue sections were cut, and subsequently dewaxed, re-hydrated and then subjected to antigen retrieval by heating in 10 mM citrate buffer in a microwave for 15 min. The sections were cooled, treated with 3% H_2O_2, blocked with 10% goat serum and then incubated overnight at 4˚C with primary antibodies against Bcl-2 (1:100 dilution, Santa Cruz Biotechnology). Negative control was incubated with an equivalent volume of diluent solution alone. After 3 washes with PBS, the standard streptavidin-biotin-peroxidase complex technique using sequential 20 min incubation with biotinylated goat anti-mouse IgG (Sigma, USA) and peroxidase-labeled streptavidin (Invitrogen, USA) was performed. We used 3,3'-diaminobenzidine (DAB) as a substrate chromogen solution for the development of peroxidase activity. Hematoxylin was used for nuclear counterstaining, then the sections were mounted and coverslipped. Images were captured with a microscope (BX51, Olympus, Japan).

Transmission electron microscopic examination. Tumor tissues from mice were prefixed in 2.5% glutaraldehyde, post-fixed in 1% osmic acid, dehydrated in gradient acetone and embedded in the resin. Ultrathin sections were cut, stained with lead citrate and assessed for the morphological changes under a transmission electron microscope.

Statistical analysis. All experiments were performed in triplicate and data are expressed as the means ± SD. Statistical analyses were conducted with the Student's t-test and

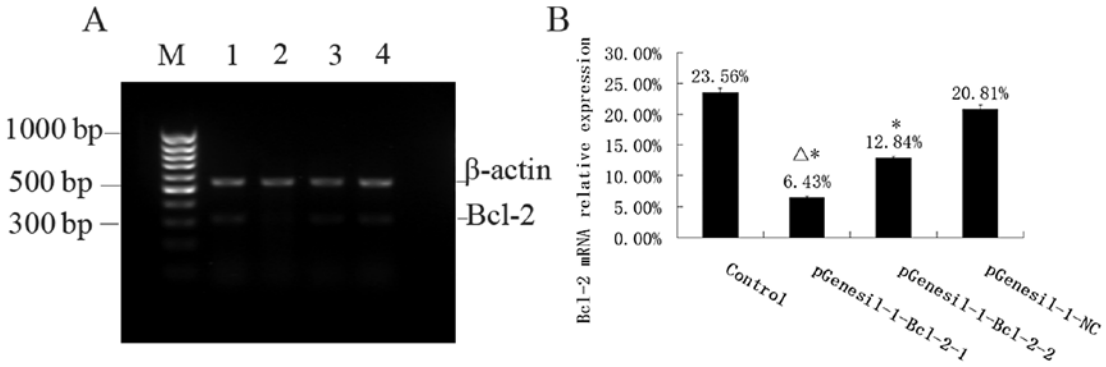

Figure 4. Bcl-2 mRNA expression detected by RT-PCR. (A) RT-PCR analysis of Bcl-2 mRNA expression in stable Raji cells transfected with plasmid vectors. M, 100 bp DNA marker; 1, untreated Raji cells; 2, stable Raji cells transfected with pGenesil-1-Bcl-2-1; 3, stable Raji cells transfected with pGenesil-1-Bcl-2-2; 4, stable Raji cells transfected with pGenesil-1-NC. (B) Quantitative expression of Bcl-2 mRNA levels. Bands corresponding to Bcl-2 and β-actin were scanned and the intensity was determined by optical density measurement. Each value represents the means ± SE from triplicate determinations. *P<0.05, vs. untreated Raji cells.

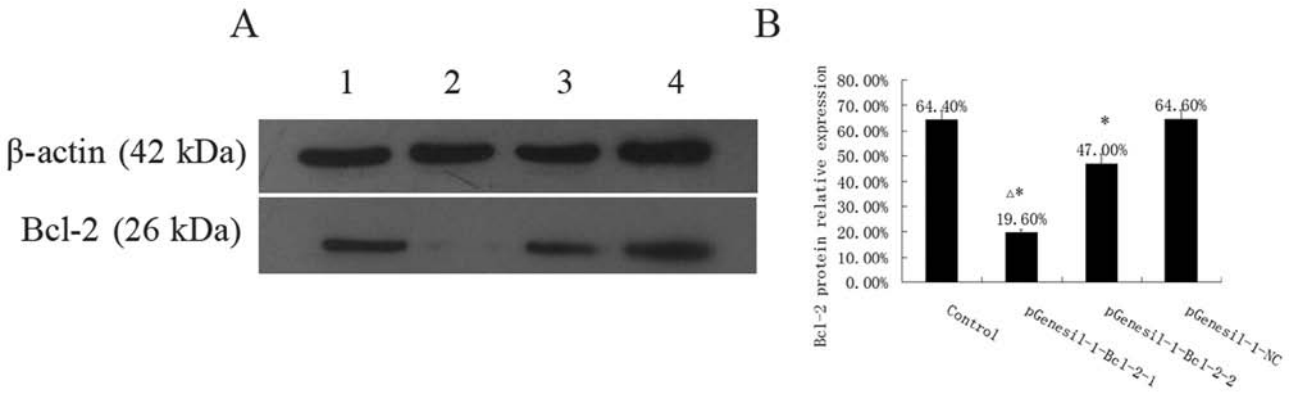

Figure 5. Bcl-2 protein expression detected by western blotting. (A) Western blot analysis of Bcl-2 protein levels. 1, untreated control group; 2, pGenesil-1-Bcl-2-1 group; 3, pGenesil-1-Bcl-2-2 group; 4, pGenesil-1-NC group; (B) quantitative expression of Bcl-2 protein levels. Bands corresponding to Bcl-2 and β-actin protein were scanned and the intensity was determined by optical density measurement. Each value represents the means ± SD from triplicate determinations. *P<0.05, vs. the untreated control group.

performed with SPSS 10.0 software. P<0.05 was considered to indicate statistically significant differences.

Results

Identification of Bcl-2 shRNA expression plasmids. Since the restriction site of enzyme *Pst*I in plasmid pGenesil-1 (Fig. 1) was replaced by the inserted DNA sequence, the reconstructed plasmids could not be digested by *Pst*I. In the inserted target gene template DNA an *Eco*RI enzyme digestion site was designed between *Bam*HI and *Hin*dIII. The pGenesil-1 plasmid itself carried an *Eco*RI enzyme digestion site. If the insertion was correct, a band approximately 400 bp should be cut off by *Eco*RI. The pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2, pGenesil-1-NC plasmids were digested by restriction enzyme *Pst*I and *Eco*RI, respectively. Agarose gel electrophoresis (1.0%) showed that *Eco*RI digestion produced a fragment of 400 bp, while *Pst*I digestion did not (Fig. 2A). DNA sequencing confirmed that the plasmids were reconstructed successfully (Fig. 2B).

Results of transfection. Twenty-five days after G418 selection, stable transfection efficiency of recombinant plasmid in Raji cells was examined by coexpressing enhanced green fluorescent protein (EGFP). When the cells were examined under a fluorescence microscope after stable transfection, >95% of cells transfected with shRNA showed fluorescence in total cells and 0.25% in control. The results showed a high efficiency of shRNA transfection (Fig. 3).

Inhibition of mRNA expression by Bcl-2 shRNA. The mRNA expression intensities of Bcl-2 genes, inhibited by specific Bcl-2 shRNAs in the Raji cells, were analyzed by semiquantitative RT-PCR. The mRNA levels were normalized by internal control β-action (Fig. 4). The rate of Bcl-2/β-actin mRNA was 23.56±0.68%, 6.43±0.25%, 12.84±0.33%, and 20.81±0.70% for the control, the pGenesil-1-Bcl-2-1, the pGenesil-1-Bcl-2-2 and the pGenesil-NC group, respectively. The statistical analysis showed that Bcl-2 mRNAs of Raji cells in the pGenesil-1-Bcl-2-1 and the pGenesil-1-Bcl-2-2 group were reduced significantly, compared with those of the control group (P<0.05). The inhibition rate reached 72.71 and 45.50% in the pGenesil-1-Bcl-2-1 and the pGenesil-1-Bcl-2-2 group, respectively. However, the pGenesil-NC group showed no significant inhibition for Bcl-2 mRNA expression (P>0.05, vs. control).

Knockdown of Bcl-2 protein expression by Bcl-2 shRNA. To confirm whether the inhibition of Bcl-2 mRNA by Bcl-2

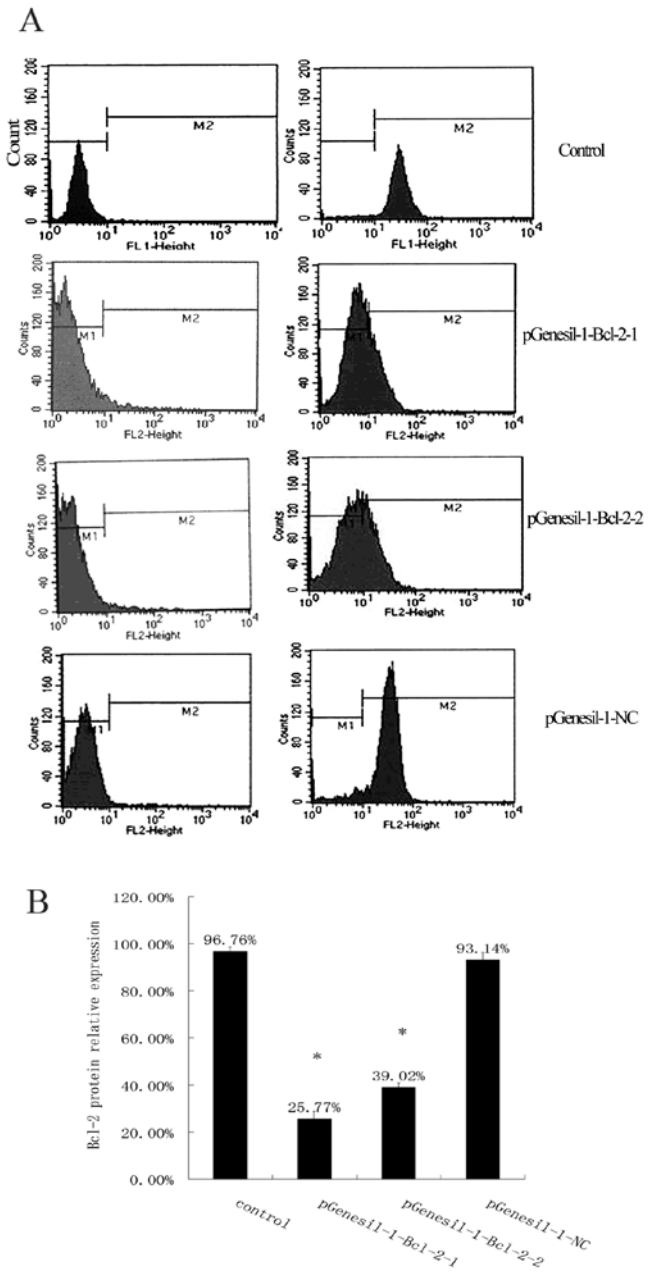

Figure 6. Bcl-2 protein expression detected by FCM. (A) FCM analysis of Bcl-2 protein levels. (B) Quantitative expression of Bcl-2 protein levels. 1, untreated control group; 2, pGenesil-1-Bcl-2-1 group; 3, pGenesil-1-Bcl-2-2 group; 4, pGenesil-1-NC group. Data are the means ± SE and analyzed by bars with standard error. *P<0.05 vs. untreated Raji cells.

specific shRNA expressing plasmid influences Bcl-2 protein expression, Bcl-2 protein levels in Raji cells after stable transfection with Bcl-2 shRNA expressing plasmids were evaluated by western blotting. As shown in Fig. 5, the Bcl-2 protein levels were 64.40±3.58, 19.60±1.14, 47.00±3.74, and 64.60±3.65% in the control, the pGenesil-1-Bcl-2-1, the pGenesil-1-Bcl-2-2 and the pGenesil-1-NC group, respectively. Protein levels of Bcl-2 in the control and the pGenesil-NC group were quite similar (P>0.05), but reduced by 69.57 and 27.02% in the pGenesil-Bcl-2-1 and the pGenesil-Bcl-2-2 group respectively (P<0.05 vs. control). We then further quantified the amount of Bcl-2 protein

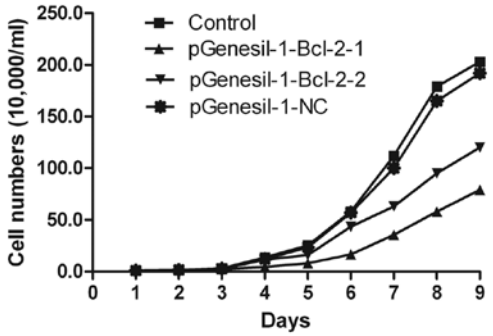

Figure 7. Curves of Raji cell proliferation. The MTT assay showed that the growth of cells transfected with pGenesil-1-Bcl-2-1 and pGenesil-1-Bcl-2-2 was suppressed as compared with untreated control cells (P<0.01) and the difference between the untreated control and the pGenesil-1-NC group had no statistical significance (P>0.05).

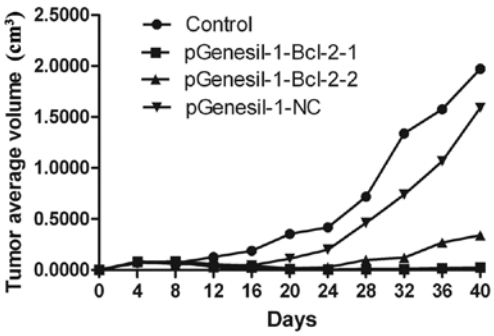

Figure 8. Raji cells stably transfected with pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2, pGenesil-1-NC and untreated control were injected into nude mice. The tumor volumes were recorded in curves.

expression in each group by using FCM (Fig. 6). The results showed that Bcl-2 protein levels were 96.76±1.83, 25.77±3.12, 39.02±1.97 and 93.14±3.14% in the control, the pGenesil-1-Bcl-2-1, the pGenesil-1-Bcl-2-2 and the pGenesil-1-NC group, respectively. Consistent with the western blot results, the statistical analysis showed that the expression of the Bcl-2 protein in Raji cells was downregulated significantly after stable transfection with Bcl-2 shRNA (P<0.05, vs. control) and the inhibition rates were 73.37 and 59.67%, respectively (P<0.05, vs. control). As expected, there was no difference in the reduction of Bcl-2 protein expression between the control and the pGenesil-1-NC group (P>0.05). The above results indicated that Bcl-2 shRNA significantly decreased the Bcl-2 protein expression levels in Raji cells following stable transfection.

Inhibition of Raji cell proliferation by Bcl-2 shRNA. Cell proliferation was measured by counting the number of viable cells using trypan blue staining. As shown in Fig. 7, when Raji cells were stably transfected with pGenesil-1-Bcl-2-1 and pGenesil-1-Bcl-2-2, respectively, the growth of the cells was suppressed as compared with untreated control cells (P<0.01). However, no statistical significance was found between the untreated control and the pGenesil-1-NC group (P>0.05). Raji cell growth was inhibited at a significantly higher rate in the pGenesil-1-Bcl-2-1 group than in the pGenesil-1-Bcl-2-2 group at different times (P<0.05).

Table II. Effect of Bcl-2 shRNA on tumor growth in nude mice (mean ± SD).

Groups	n	Days of tumor formation	Size of tumor (cm^3)	Weight of tumor (g)
Untreated control	6	8.2±1.2	1.9712±0.3309	0.7810±0.2288
pGenesil-1-Bcl-2-1	6	24±2.3	0.0238±0.0142[b]	0.0533±0.0058[b]
pGenesil-1-Bcl-2-2	6	20±2.0[a]	0.3397±0.0581[b]	0.2053±0.0200[b]
pGenesil-1-NC	6	10±1.5[a]	1.5910±0.2480	0.7533±0.0706

Tumor tissues were excised 40 days after subcutaneous inoculation in mice. The size and the weight of the tumors were measured. [a]P<0.05, [b]P<0.01, compared with either the untreated or the pGenesil-1-NC group.

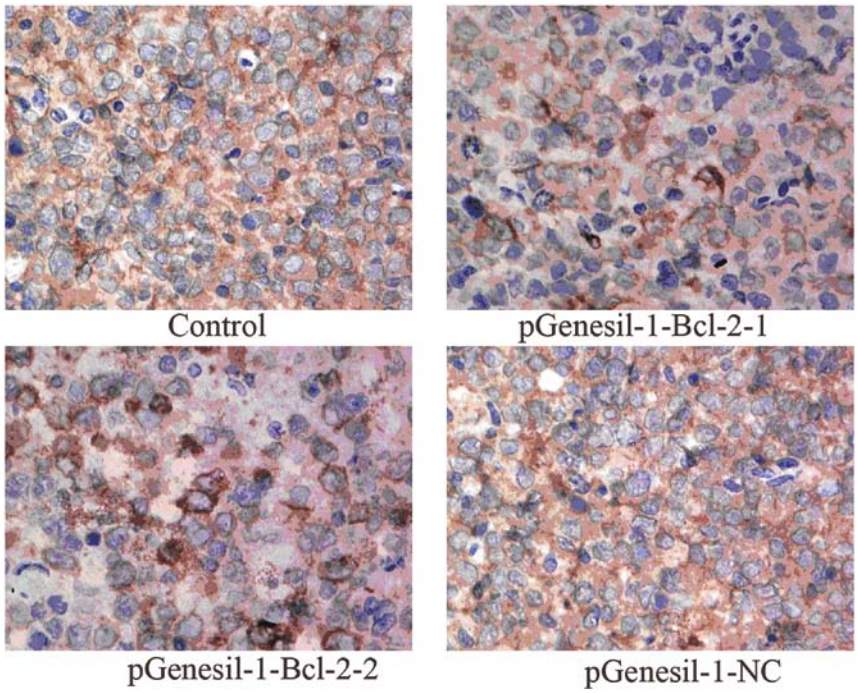

Figure 9. Bcl-2 protein expression of B-cell lymphoma tissues was detected. Bcl-2 protein positive cells were stained by immunohistochemistry (original magnification x400).

Inhibition of in vivo tumor growth by Bcl-2 shRNA. To determine the potential effects of Bcl-2 shRNAs on the inhibition of the growth of Raji cells *in vivo*, equal numbers ($3x10^7$ cells/ml) of Raji cells stably transfected with pGenesil-1-Bcl-2-1, pGenesil-1-Bcl-2-2, pGenesil-1-NC and untreated control were injected into nude mice subcutaneously. The growth of tumors was measured every four days. Forty days after injection, mice were sacrificed and the weights of tumors were recorded. As shown in Table II and Fig. 8, untreated Raji cells and those of the pGenesil-1-NC group grew rapidly, resulting in palpable tumors 8-12 days following injection. By contrast, tumor formation was significantly slower after inoculation of pGenesil-1-Bcl-2-1 or pGenesil-1-Bcl-2-2 clone (P<0.01). The tumors were significantly smaller than those in either the untreated control or the pGenesil-1-NC group (P<0.05). These results indicated that Bcl-2 shRNA-mediated Bcl-2 downregulation exerted a strong antitumoral effect *in vivo* on B-cell lymphoma.

Tumor tissues from mice were excised and subjected to immunohistochemistry staining. As shown in Fig. 9, the microscopic examination of stained tumor sections showed that Bcl-2 expression was strongly detected in the untreated control and the pGenesil-1-NC group, respectively. However, weak immunoreactivity was observed in the pGenesil-Bcl-2-1 and the pGenesil-1-Bcl-2 group, respectively. Bcl-2 expression in the control and the pGenesil-NC group were markedly higher than in the pGenesil-1-Bcl-2-1 and the pGenesil-1-Bcl-2 group (both P<0.01), suggesting Bcl-2 shRNA remarkably downregulated Bcl-2 protein expression on B-cell lymphoma *in vivo*.

Morphological change under transmission electron microscopy. With the help of the trans-nuclear membrane, distributed nuclear chromosome, distinct organelle, big nuclei and mission electron microscope, we found that the normal Raji cell had intact cell membrane and excessive nuclei divi-

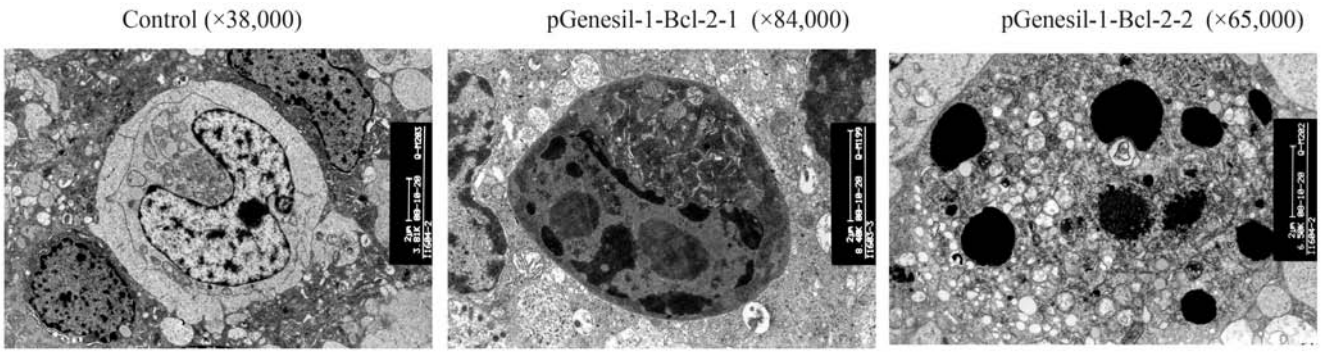

Figure 10. Effects of Bcl-2 shRNA on tumor apoptosis *in vivo*. Ultrathin sections were cut, stained with lead citrate, and assessed for the morphological changes under a transmission electron microscope.

sion, which indicated that the Raji cell was relatively highly malignant. When the Raji cells were transfected with Bcl-2 shRNA, some changes, such as apoptosis, cell shrinkage, separation from neighboring cells, plasma condensation, plasma vacuolation, karyopyknosis, margination of condensed chromatin and membrane-bound apoptotic bodies were observed (Fig. 10).

Discussion

The worldwide incidence of lymphoma is increasing and lymphoma accounts for approximately 3-4% of all types of cancer (11). The conventional treatments including surgery, chemotherapy, radiation therapy and immunotherapies do not achieve satisfactory effects due to drug resistance and lymphoma recurrence. In addition, many conventional treatments usually lead to toxicity that affects therapeutic outcome and quality of life. Thus, it is urgent to find a novel approach to overcome these short comings in cancer therapy. With the development of molecular biotechnology, gene therapy becomes a new potential approach for the treatment of cancer, as it has a highly efficient, specific effect and only slight drug resistance. To date, RNAi has attracted much attention. RNAi is one of the most commonly used approaches for genes targeting cutting edge technology (12). RNAi is mediated by small interfering RNAs of approximately 21 nucleotides or by continually expressed short hairpin RNAs that are cut into siRNAs by Dicer (13,14). Then, the produced siRNA is incorporated into nuclease complex, forming the RNA-inducing silencing complex, which degrades mRNA containing a sequence homologous to that of the small RNA fragment (15-19). As a post-transcriptional gene silencing mechanism, RNAi has been demonstrated to have prospects for cancer therapy.

The development of B-cell lymphoma is correlated with multiple genes. Among these genes, Bcl-2 plays an important role (20). Bcl-2 is a member of the Bcl-2 family that is key in the regulation of the intrinsic pathway by controlling mitochondrial membrane permeability and the release of the proapoptotic factor cytochrome *c*. Bcl-2 overexpression has been demonstrated in B-cell lymphoma (21,22). Bcl-2 overexpression has been reported to be involved in the progression of tumors (23,24). Xu *et al* noted that pGenesil-1-Bcl-2 shRNA could significantly inhibit Bcl-2 expression, it suppressed the growth of human bladder cancer cells T24 and induced apoptosis of the cells (25). Lei *et al* (26) demonstrated that Bcl-2 shRNA reduced the level of Bcl-2 mRNA and Bcl-2 protein expression in HL-60 cells and induced cell apoptosis. Therefore, specific downregulation of Bcl-2 may be a potential therapeutic strategy against human cancer.

To investigate whether Bcl-2 shRNA could suppress the development of Raji cells from B-cell lymphoma by the above RNAi method, we constructed two types of Bcl-2 shRNA and stably transfected them into Raji cells. Then, we detected Bcl-2 mRNA and its corresponding protein expression in the pGensil-1-Bcl-2-1 and the pGensil-1-Bcl-2-2 group. Meanwhile, Raji cell proliferation was successfully inhibited by Bcl-2 shRNA, compared with the control group ($P<0.05$). In order to further investigate whether Bcl-2 shRNA mediated downregulation *in vivo*, we chose 4-week-old BACB/C nude mice lacking T cell immune function as the animal model. When compared with the pGenesil-1-NC and the untreated Raji cell group, tumor formation was significantly slower after inoculation of pGenesil-1-Bcl-2-1 or pGenesil-1-Bcl-2-2 clone ($P<0.01$) and the tumors were significantly smaller ($P<0.05$). The results of immunohistochemistry showed that Bcl-2 shRNA markedly reduced the expression of Bcl-2 protein in Raji cells and the transmission electron microscope demonstrated that special Bcl-2 shRNA successfully induced Raji cell apoptosis. These data indicated that Bcl-2 shRNA-mediated Bcl-2 downregulation blocked the transduction of survival signals and trigger apoptosis, as previously reported (27), and displayed a strong growth inhibition on B-cell lymphoma *in vitro* and *in vivo*. Bcl-2 shRNA could be applied for treatment of tumors with overexpression of Bcl-2. However, we observed that the inhibition effect of the pGenesil-1-Bcl-2-1 group was better than that of the pGenesil-1-Bcl-2-2 group. The possible reasons were that the nucleotides 544-562 of mRNA contained few cytosine and guanine and its space structure was easy to open compared to the nucleotides 1009-1027. However, the underlying mechanism requires further examination.

In this study, we also observed that BCL-2 protein levels were reduced by 69.57 and 27.02% in the pGenesil-Bcl-2-1 and the pGenesil-Bcl-2-2 group compared with untreated control by using western blot analysis. The inhibition rates were accordingly lower than 73.37 and 59.67% by flow cytometry. We considered a possible cause that detectability and accuracy of western blot analysis were different from flow cytometry.

However, the two test methods consistently indicated that Bcl-2 shRNA was able to decrease the Bcl-2 protein expression levels in Raji cells.

In summary, our study indicated that Bcl-2 performed a fundamental role in the progression of the tumor, and plasmid-mediated Bcl-2 shRNA inhibited the growth and apoptosis of human B-cell lymphoma *in vitro* and *in vivo*. RNAi may have potential therapeutic utility in cancer and other diseases.

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