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Original Research

Lentiviral-mediated *BCL2* gene knockdown using comparative microRNA adaptive shRNAs

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Abstract: B-cell lymphoma 2 (*BCL2*) family proteins play a critical role in tuning cell death processes. Almost in half of all human cancers, a dysregulation in *BCL2* family gene expression has been shown which made it an impressive target for human gene therapy as a novel approach in cancers. In this study we will optimize lentiviral-mediated RNA interference (RNAi), recombinant lentiviruses accommodating anti-*BCL2* micro adaptive short hairpin RNAs (shRNAs), to downregulate *BCL2* in human embryonic kidney 293T (HEK293T) cells to produce stable cell lines. We tested 4 different Dharmacon™ GIPZ™ shRNAmir lentiviral vectors targeting *BCL2* in different positions and a pGIPZ non-silencing shRNAmir lentiviral vector (as a negative control). Lentivirus packaging was performed by the calcium phosphate precipitation method. HEK293T cells were transduced by each type of recombinant lentiviruses individually and selected by puromycin within 10 days. The relative mRNA level and protein expression were assayed by using real-time polymerase chain reaction (PCR) technic and western blotting, respectively. Lentivirus (LV) packaging was performed in high efficiency (transfection rate was > 90%). Recombinant viruses of 4 expression vector addition to a control vector were produced then transduced to HEK293T cells successfully. All the 4 cell groups showed a significant down regulation of *BCL2* gene (~90-95%) at mRNA level compared to the control group ($p<0.01$) but differences between silenced groups were not significant ($P > 0.05$). We showed that the lentivirus-mediated RNAi technique is an efficient method to establish HEK293 cell lines with stable down-regulation of *BCL2* gene.

Key words: *BCL2*; Lentivirus; RNA interference; HEK293 Cells; Genetic Transductions.

Introduction

BCL2 family is involved in determining the fate of cell life. About thirty years ago, *BCL2*, the founding member, was discovered during a chromosomal rearrangements analysis in follicular B-cell lymphomas (1). First, it was suspected to be an oncogene because of its role in cell proliferation but later understood to inhibit cell death and other members introduced in investigations about apoptosis in *C. elegans* worm(2). The family comprises about 20 homologues members that are divided into three groups: pro-apoptotic, BH3-only and antiapoptotic proteins (3). They interact in a vast complex signaling pathway as regulators of all kinds of cell death processes including apoptosis, necrosis, and autophagy. An imbalance between anti and pro apoptotic members (for instance *BAX* and *BCL2*) or overexpression of anti-apoptotic members alone (like *BCL2)* due to genetic and epigenetic events may lead to disturbed cell death settings and subsequent exaggerated cell proliferation, a condition that is known as cancer (4).

Cancer is the cause and effects of defective apoptosis in which the overexpression of *BCL2* is a common event. It blocks cell death signals from several stimuli like lack of growth factors, hypoxia, and oxidants overload, radiation and anti-cancer drugs that results in unusual cell proliferation and failure of conventional therapies for tumor eradication (5). Myriad studies have shown a strong correlation between BCL2 protein levels and poor prognoses in numerous cancers such as melanoma (6), colorectal carcinomas (7) breast (8, 9), prostate (10), lung (11), and especially hematological cancers like non-Hodgkin's lymphomas (12). In fact, BCL2 is in the downstream signaling pathway of all anti-cancer drugs and also the escape point for tumor cells to evade chemotherapy. These data are appealing enough for researchers to focus on *BCL2* as a target for gene silencing and drug development.

In this regard, RNAi systems have been exploited successfully to down regulate cancer-related culprit genes (13). RNAi is an appealing issue in the field of gene regulatory systems. The response mediated by double stranded RNAs (dsRNAs) results in cognate gene silencing at the post-transcriptional levels (PTGS). Synthetic siRNAs and vectors transcribing small hairpin RNAs (shRNAs) can be used to inhibit gene expression (14). Although mammalian cell transfection by siRNAs has indicated remarkable responses, the most basic drawback is its transient nature. Since, shRNAs are introduced to cells by plasmid or viral-based vectors,

gene expression can be down regulated as far as they are transcribed. So it is a preferable method when stable expression network changes are desired (15). Among different delivery systems for shRNAs, lentiviral-based shRNA expressing vectors seem to be more popular. They are the main delivery systems in emerging technologies like CAR-T cell and CRISPR-Cas lentiviruses. Lentiviral vectors are efficient, easy to use and capable of transducing dividing and non-dividing cells and also a plethora of shRNAs libraries in lentiviral vectors have been established. This has accelerated research trends focusing on modulating gene expression in cell cultures as well as in animals in order to concur unruly diseases (16, 17)

In this research, we chose HEK293T cells for lentivirus packaging and transduction. The cell line has been used largely in cell biology researches because of simple growth and maintenance, highly efficient transfectibility and protein production, flawless translation and processing of proteins. These facilitate gene expression manipulating to analyze protein functions and effects of new drugs (18). We packaged 4 different lentiviruses containing anti-*BCL2* shRNAmirs and a scrambled-shRNAmir lentivirus as negative control. Then transduced HEK293T cells in 4 groups to target *BCL2* in different positions and a group for non-silencing shRNA. We assayed mRNA and protein levels of *BCL2* using real-time PCR and western blot test then compared efficiencies to provide a potent downregulation of *BCL2*. In addition, we exploited the genome integrating quality of lentiviruses in order to create a stable cell line.

Materials and Methods

Cell culture

HEK 293T (human embryonic kidney, ATCC® CRL-3216™) gifted from Mede (Bio-economy Company, Iran) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10 % fetal bovine serum (FBS, Gibco, USA), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and incubated at 37 ºC in a humidified 5% CO2 atmosphere.

Lentiviral shRNAmir transfer vectors

Four pGIPZ shRNAmir Lentiviral vectors targeting *BCL2* gene and a pGIPZ non-silencing shRNAmir lentiviral control vector were purchased from GE Healthcare Dharmacon Inc. Vectors contain Turbo GFP and puromycin-resistant gene as reporter and selection marker, respectively, as well as a microRNA-adapted shRNA against *BCL2* gene and internal ribosome entry site (IRES). Expressions are controlled by CMV promoters. Sequences of these multi comparative anti-BCL2 shRNAmirs are given in table 1.

Recombinant lentivirus production

Each lentiviral vector was co-transfected with psPAX2 and pMD2.G plasmids (both were gifts from Didier Trono (Addgene plasmid # 12260 and # 12259)) in HEK-293T cells, using the standard calcium phosphate precipitation method according to Tronolab protocol (19). The day before LV packaging, we plated 2.5×10^6 HEK293T cells in each 10cm plate and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10 % fetal bovine serum (FBS, Gibco, USA). On the day of transfection, each shRNA cassettes including: V2LHS_111806, V2LHS_111810, V3LHS_375361, V3LHS_410736 and a scrambled negative control were mixed with appropriate concentrations of psPAX2 and pMD2G into HEK293T. After 8 hours media was replaced with fresh DMEM 10 then every 8-12 hours media containing viruses was collected for 3 times. The efficiency of transfection was checked under fluorescent microscopy. Pooled supernatants were centrifuged at 2000 rpm, 4 °C for 5 minutes and filtered using a 0.22-µm filter unit then stored at-80°C.

Virus titration

First, 5 x 10^5 cells/well were seeded in a 12 wells plate with 1 ml of DMEM 10 medium. Next day, medium were removed. Five serial dilutions of virus supernatants were prepared from 1µl to 10^{-4} µl. Volumes were reached to 500µL by DMEM 10 and added to wells in duplicate. The plate was incubated at 37°C, 5% CO2. On day 5 cells were harvested and sent for flow cytometry in order to analyze cells for GFP expression. The dilution that resulted in 1-20% GFP positive was chosen and virus titration was calculated using the standard equation:

"Titer (HEK293T transducing units/ml) = (Number of target cells on day 1) × (% of GFP-positive cells/100) / volume of supernatant (ml)"

Transduction of HEK293T cells

For transduction, on day one ~1.2 x 10^6 cells/well were seeded in 2mL of DMEM 10 medium onto 6-well culture plates and incubated overnight at 37°C, 5% CO2. Next day, lentiviral supernatants were gently added to each well with a multiplicity of infection (MOI) of 5. The day after, the virus media was removed and replaced with normal DMEM media supplemented with 10% FBS and 1% PenStrep. The percentage of transduction detected by observing cells under fluorescence microscopy after 96 hours and a stable cell line acquired by treating with puromycin (1.5mg/ml) within 10 days.

Real-time-PCR detection BCL2 mRNA

To confirm *BCL2* gene silencing and compare the efficiency of *BCL2* down regulation, total RNA of each stable cell line was extracted using RNeasy® Mini kit

Table 1. Sequences of anti-sense fragments for shRNAs.

Name	Anti-sense sequences	Target site
V2LHS_111806	TGAATATACACAATCAGGG	1998-2016
V2LHS_111810	TGTCACACAAGGTTCTTCG	5356-5374
V3LHS_375361	ACTTCATCACTATCTCCCG	527-545
V3LHS_410736	TGAACAACAACAAAAGACA	3868-3886
Non-silencing	ATCTCGCTTGGGCGAGAGTAAG	-

(QIAGEN) and reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The resulted cDNA amplified during real-time PCR process. Primers were 5′-CCTGTGGATGACTGAGTACC-TGA-3′ (forward (and 5′-ACTGAGCAGAGTCTTCA-GAGACAG -3′ (reverse). The internal control was actin and primers designed as 5′- AGCCTCGCCTTTGCCGA -3′ (forward) and 5′- CTGGTGCCTGGGGCG-3′ (reverse). The qRT-PCR was performed with QuantiTect SYBR Green RT-PCR Kit (QIAGEN) according to the manufacturer's instructions. The amplification program contained 40 cycles of denaturation at 94˚C for 15sec, annealing at 60˚C for 30sec, and extension at 72˚C for 30 sec. Fluorescence data were collected at the extension step and analyzed by the REST program. Expression of treated samples was calculated by normalizing with actin gene, the calibrator. All were run in triplicate.

Western blot analysis

Cells lysed in 200 µl of RIPA buffer (Thermo Fisher Scientific, IL) supplemented with the protease inhibitor. Then centrifuged at 16,000 ×g for 20 min at 4˚C. After determining protein concentrations 12% SDS-PAGE was performed to separate proteins followed by transferring onto nitrocellulose membranes to immunoblot with antibodies against BCL2 and GAPDH (Santa Cruz; 1/1000) as an internal control. Finally incubated with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Abcam; 1/10,000) for 1h at room temperature. Then protein bands were visualized by ECL and analyzed by ImageJ Software.

Statistical analysis

Data were indicated as mean ± SD. Comparisons among cell groups were accomplished with one-way analysis of variance (ANOVA) test using SigmaPlot software. Values of $p<0.05$ were considered statistically significant.

Results

Preparation and confirmation of vectors

Since we had delivered lentiviral vectors in form of transformed bacteria, first they were proliferated and plasmids were extracted using GeneJET Plasmid Midiprep Kit (Thermo Fisher Scientific, USA) follow the manufacturer's instructions then confirmed by SalI enzyme (data not shown).

Lentivirus packaging

Production of four recombinant lentiviruses and a scrambled vector were done successfully and verified by tracing tGFP 24, 48 and 72 h after transfection in all cell groups using fluorescence microscopy. As shown in Figure1 the rate of transfection was more than 90% in all groups.

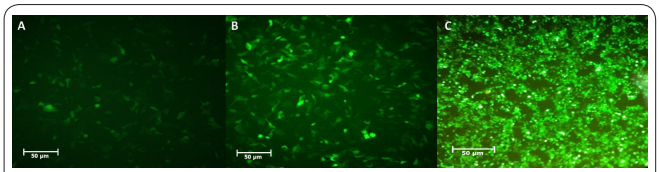

Figure 1. Lentivirus packaging in HEK 293T. Fluorescent images have taken 24(A), 48(B) and 72(C) hours after transfection.

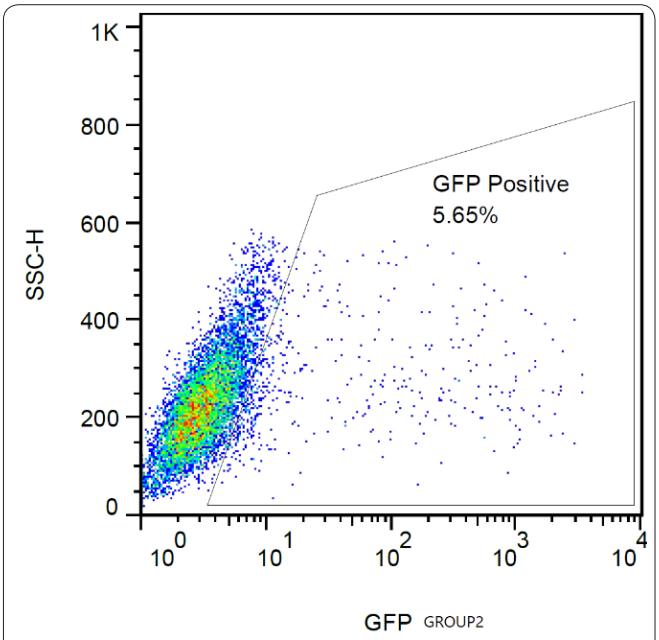

Figure 2. Titration of lentiviruses using flow cytometry. The Chosen dilution for titer calculations was 10^{-2} µl (group2) yielding 5.65% GFP positives.

Titration

The dilution of 10^{-2} µl, was chosen for titer calculation because it was yielded 5.65% GFP positives (Figure 2). Data was placed in the formula and virus titer was around 2×10^7 IU/mL in almost all groups, however control negative met a higher titer.

Establishing a stable HEK 293T cell line

One day after transduction, cells grew to express green GFP under fluorescence microscopy. About 80-90% of cells were GFP positive 96hr after transduction (Figure 3.) however, after treating with puromycin the rate reached up to 100% during two weeks and stable HEK 293T cell line was achieved.

Real Time PCR assay in transduced HEK293T cells

Cell groups underwent real time PCR test to analyze gene expression of *BCL2* at the mRNA level. Results that were analyzed by REST software declared that *BCL2* expressions were significantly down regulated in all four cell groups treated with recombinant lentivirus-mediated shRNA expression vectors (~90-95%) but not in non-silencing and normal groups ($P < 0.01$). As shown in Figure 4 and confirmed by ANOVA test there was no statistical difference between silenced groups ($p > 0.05$) however, the cassette V3LHS_410736 and V2LHS_111806 were a little more efficient than others

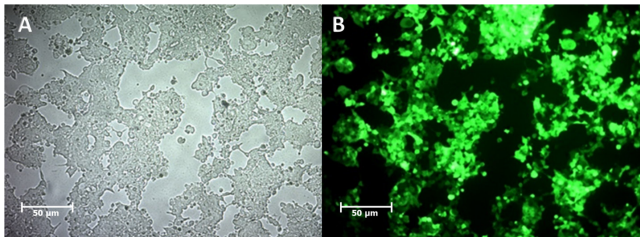

Figure 3. Transduction of HEK293 cells. Light (A) and fluorescent (B) images of transduced HEK293 cells using anti-*BCL2* pGIPZ-Lentiviral shRNAmir vector (V3LHS_410736).

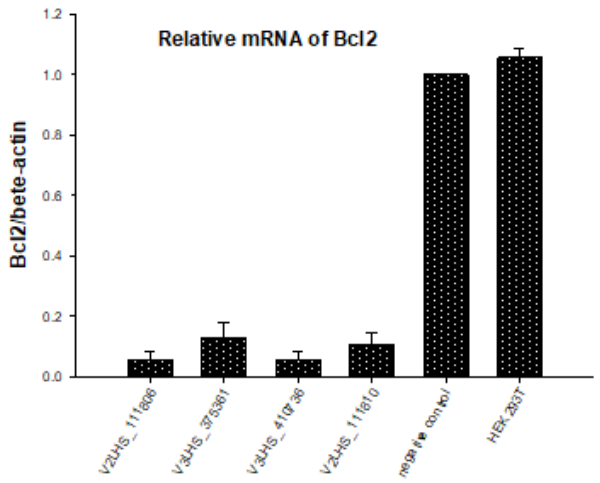

Figure 4. Relative mRNA levels of *BCL2* in transduced HEK293T cell groups. *BCL2* has been silenced significantly by four different recombinant lentiviral shRNAmir vectors compared with scrambled shRNA (negative control) and normal groups (HEK293T cells).

in *BCL2* silencing. Totally, all 4 types of recombinant viruses were strongly able to pull down *BCL2* gene expression at the mRNA level in HEK293T cells.

Western blotting assay in transduced HEK293T cells

We measured the BCL2 protein level in transduced cells by western blot test to confirm *BCL2* silencing at post-translational level. The results showed that BCL2 protein level was significantly decreased in all four groups that were transduced by recombinant lentiviruses containing anti-*BCL2* shRNAs but not in negative control and mock groups ($p<0.01$) (Figure 5A). Comparing the BCL2 levels, differences between silenced groups were not statically meaningful as seen in real time PCR analysis ($p>0.05$).

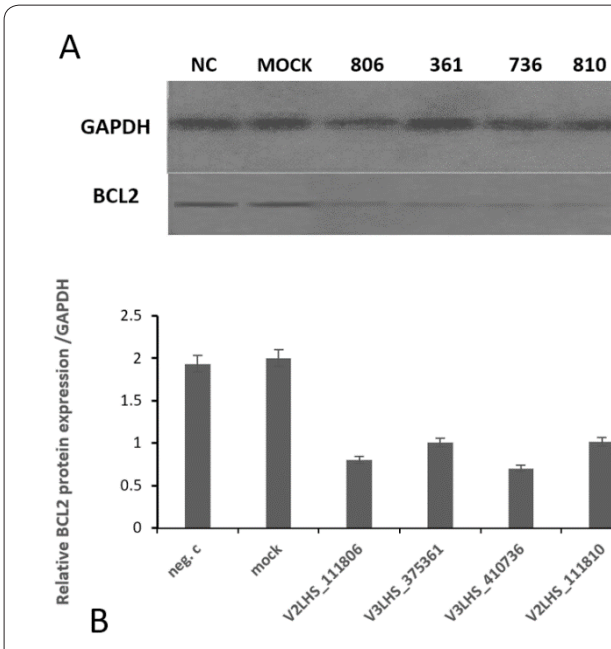

Figure 5. Quantification of BCL2 protein in HEK293T cells. **A:** Western blotting of four groups of transduced HEK293T cells by recombinant lentivirus mediated anti *BCL2* shRNAs compared with non-treated HEK293T cells(mock) and negative control(NC) transduced by recombinant lentiviruses containing scrambled shRNAs. **B:** Using ImageJ Software, relative BCL2 protein expression to GAPDH as internal control revealed a significant down regulation in four experimental groups ($p<0.01$).

Discussion

Dysregulation of *BCL2* family genes may result in faulty cell death that is among the six criteria of cancer (20). Since the first report about oncogenic feature of BCL2 by Reed et al in 1988, (21), researches have demonstrated that over expression of anti-apoptotic *BCL2* family proteins in malignancies are responsible for cell growth, resistance to anti-cancer therapies and consequently poor prognosis (22). Efforts to decrease *BCL2* levels in different cancers have been promising for cancer treatment and even cure in future (23, 24). To achieve this goal a stable gene silencing in cancer cells is favorable. By establishing stable cell lines one can properly study outcomes of gene engineering and benefit in human studies.

In the present study, we have shown that lentiviral-mediated RNAi is a potent tool to stably silence *BCL2* in HEK293T cells. Lentiviral vectors have high transfection efficiency and ability to infect different cells by natural tropism so they are considered as a natural transporter of DNA into the nucleus. They infect cells with high efficiency and large sequences can be put up. Besides, they have the ability to integrate into the host genome in a stable mode to provide a long-term protein expression which enables repeated experiments to be done with less variation due to repeated transient transfection. (25).

To optimize the RNAi system, we used 4 different Dharmacon™ GIPZ™ lentiviral vectors (Table 1) that incorporate shRNAmirs targeting different regions of *BCL2* gene. They were mir-based and under the control of CMV promoters that utilize RNA polymerase II to carry out transcription. It would minimize viral titer and reduce the possibility of concentration dependent off-target effects. They also express fluorescent protein (GFP) to track shRNAmir expression and Puromycin resistance gene as a selection marker. Redundancy in shRNAs with similar phenotype causes discrimination between target-dependent effects and non-specific effects and thus minimizes off-target activity (26). Additionally, negative control was a scrambled lentiviral-based shRNAmir. The non-silencing shRNAmir sequences contain no homology to known mammalian genes but engage RISC so reveal real gene silencing. All the constructs were miR-30 adaptive shRNAs that means the stem of the primary miR-30 transcript was replaced with gene-specific duplexes for *BCL2*. It does not affect normal miRNA maturation and exploits endogenous enzymatic processing by the RNase III Drosha, which increases subsequent Dicer recognition and specificity. The design results in producing mature siRNAs that are ten times more potent (27).

We produced the recombinant lentiviruses and transduced to HEK293T cells successfully and selected with puromycin. All the cell groups showed a significant down regulation of BCL2 compared to the negative control and normal groups that confirmed with Q-PCR and Western blot assays which demonstrated the lentivi-

rus-mediated RNAi technique is an efficient method to down regulate *BCL2* expression in HEK293T cell line. In fact, our study has used lentiviral recombinant vector on HEK293T to decrease *BCL2* expression. A similar method has been used successfully in our laboratory to target IGF1R on HEK293T (28). Yao Zhao et al. studied lentivirus-mediated *BCL2* silencing in osteosarcoma cells to enhanced chemosensitivity of drug-resistant (29). Some studies have also used lentiviral-based RNAi to silence *BCL2* in other cell lines (30, 31).

Assuming that *BCL2* has an undeniable role in cancer pathogenesis and drug resistance, it is sensible to consider *BCL2* gene therapy a promising candidate for clinical trial in future. A phase I clinical trial has been performed on *BCL2* antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma (32) and for melanoma, chronic lymphocytic leukemia (CLL), multiple myeloma and acute myeloid leukemia (AML) are in a randomized phase 3 trials (33). However, there is no study in human using a lentiviral recombinant vector to target *BCL2*.

According to our results, anti-*BCL2* shRNAmir lentiviral vectors are effective tools for *BCL2* silencing. We suggest using lentiviral vectors incorporating anti *BCL2* shRNA in human cancer cells with high *BCL2* levels such as those originated from lymphoid, myeloid, brain, skin, breast and prostate organs. Of course it would be an introduction to cancer studies in human.

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Author's contributions

Gholamreza Tavoosidana supervised the project, Kamal Yavari contributed to conception, designed the project and revised the article. Mehdi Banan contributed to conception and revised the article. Baharak Abdolhossein Zadeh worked out almost all of the technical details and wrote the manuscript. Ali Fallah helped in lentivirus production and generously provided us with his laboratory. Leila Nasehi helped in all project steps and Moloud Absalan helped in real time PCR experiment.

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