**ISSN** 1672-9145 Acta Biochimica et Biophysica Sinica 2004, **36**(11): 749–753 **CN** 31-1940/Q

# Suppression of *bcl-2* Gene by RNA Interference Increases Chemosensitivity to Cisplatin in Nasopharyngeal Carcinoma Cell Line CNE1

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**Abstract** To explore the effect of suppressing BCL-2 expression using RNA interference (RNAi) technique in nasopharyngeal carcinoma cell line CNE1. CNE1 cell lines stably expressing shRNAs targeted *bcl-2* and GL3 gene were established and gene expression inhibition was assessed by Western blotting analysis. The effect of suppressing *bcl-2* by RNAi on cell growth was studied, the apoptosis induction and the sensitization of CNE1 cells to cisplatin were quantified by MTT assay and flow cytometry. The results showed that: stable transfection of CNE1 cells with vectors expressing shRNAs against *bcl-2* decreased the expression of BCL-2 protein; suppression of BCL-2 expression did not affect cell proliferation but could increase the chemosensitivity to cisplatin in CNE1 cells. This will help physicians to make some clinical trials of gene therapy on nasopharyngeal carcinoma by RNAi.

**Key words** RNA interference; nasopharyngeal carcinoma; *bcl-2*; cisplatin

RNA interference (RNAi) is a powerful tool for the analysis of gene function in many organisms like *Caenorhabditis elegans* [1], *Drosophila* [2,3], *Trypanosoma brucei* [4], planaria [5], and fungi [6], by introducing double-stranded RNA into cells and leading to the sequence-specific destruction of endogenous RNAs that match the dsRNA. However, in most mammalian cells this would provoke a strong cytotoxic response, and the nonspecific global shutdown of protein synthesis is induced by dsRNA longer than 30 bp and masks any sequence-specific effects that might occur from the RNAi pathway [7,8]. So the application of RNAi technique was limited in mammalian cells.

In 2001, Tuschl and coworkers [9] made the remarkable observation that transfection of synthetic 21 nt siRNA duplexes into mammalian cells effectively inhibited endogenous gene expression in a sequence-specific manner. These siRNA duplexes are too short to trigger the nonspecific dsRNA responses, but they still cause the destruction of complementary RNA sequences. DNA vectors to mediate RNAi by expressing small hairpin RNA (shRNA) from RNA polymerase III promoters are constructed which can induce stable, long-term, and highly specific gene silencing [10–15]. These findings have opened a broad new avenue for the analysis of gene function and gene therapy.

In this study, DNA vector pSUPER.retro expressing shRNAs corresponding to the endogenous *bcl-2* gene is introduced into a nasopharyngeal carcinoma (NPC) cell line CNE1 to explore the potency of RNAi. *bcl-2* gene encodes an anti-apoptotic protein which can suppress the programmed cell death. Our results demonstrate that stably suppressing BCL-2 expression do not affect cell proliferation, but can induce a significant sensitization of CNE1 cells to cisplatin. It suggests the potential utility of the RNAi phenomenon as a novel therapeutic approach to nasopharyngeal carcinoma.

## Materials and Methods

### Design of shRNA

Because not every gene-targeted shRNA can inhibit the expression of targeted gene, we choose two sequences in *bcl-2* cDNA as our target sites using siRNA design software downloaded from internet (http://www.oligoengine.com). The shRNA designed against luciferase GL3 gene was used as control. BLAST search against EST libraries

Received: July 12, 2004 Accepted: September 10, 2004
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was performed to ensure that no other human gene was targeted. DNA template encoding DNA were designed as follows: 19-nt target sequence as sense strand followed by a 9-nt spacer and complementary antisense strand and then six repeats of T as termination signal. The shRNAs were subcloned into the pSUPER.retro (Oligoengine, USA) with human H1-RNA promoter between the *Hin*dIII and *Bgl*II restriction sites. psB1, psB2 and psGL3 were verified by DNA sequencing.

### Cell culture and transfection

The nasopharyngeal carcinoma cell line CNE1 was passaged in RPMI 1640 medium supplemented with 15% calf bovine serum, 100 u/ml penicillin and 100 μg/ml streptomycin, in humidified 5% $CO_2$/95% atmosphere at 37 °C.

Twelve hours before transfection, cells were seeded onto 6-well plates with antibiotics-free growth medium at a density of $3\times10^5$ cells/well, so that the confluence would reach approximately 80%–90% at the time of transfection. Cells were transfected with 4 μg/well of shRNA vector targeting *bcl-2* or luciferase GL3 gene using the Lipofectamine™ 2000 reagent (Invitrogen, USA) following the protocol provided by the manufacturer. Forty-eight hours later, cells were harvested and passaged into three wells, and clones stably expressing shRNA were selected using 8 μg/ml puromycin (Sigma, USA).

### Western blotting analysis

Cells were lysed in 50 μl lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5 mM DTT, 2% SDS) on ice for 30 min, and the resulting lysates were cleared by centrifugation. Proteins were resolved by 10% SDS-PAGE and electroblotted onto nitrocellulose membrane, blocked by 5% skim milk, and probed with anti-BCL-2 (Santa Cruz, USA) or anti-α-tubulin (Santa Cruz, USA) antibody. Following incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Amersham Pharmacia Biotech), immunoblots were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech).

### MTT assay

MTT assay was performed to assess the silencing effect of *bcl-2* on cell proliferation and chemosensitivity of CNE1 cells to cisplatin. Cells were plated in 96-well plate at a density of $1\times10^4$ cells/well. 4 h or 48 h later, cells were stained with 20 μl sterile MTT dye (5 mg/ml, Sigma, USA) for 4 h at 37 °C, then culture medium was removed and 200 μl of DMSO was added and thoroughly mixed for 10 min. Spectrometric absorbance at wavelength of 570 nm was measured on a microplate reader (ELx800, Bio-Tek, USA). The value of $A_{490}$(48 h) vs. $A_{490}$(4 h) was calculated as cell proliferation index. Each group contained five wells. To assess chemosensitivity to cisplatin, the cells cultured for 24 h were incubated with different concentrations of cisplatin for another 48 h. Then cells were treated with MTT as we just described. The value of $[A_{490}(\text{cisplatin}^+)/A_{490}(\text{cisplatin}^-)]\times100\%$ indicated the cell survival index.

### Flow cytometry

Cell apoptosis and cell cycle profiles were analyzed by flow cytometry. $1\times10^6$ cells were collected and washed with phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol and stored at 4 °C. After resuspended in PBS containing 100 μg/ml RNase A and 20 μg/ml propidium iodide (PI) for 30 min, samples were analyzed by flow cytometry (FACS Calibur, BD, USA). The cell cycle phase distribution was calculated from the resultant DNA histogram using Mod Fit LT software. The apoptotic cells were observed as a subdiploid peak.

### Statistical analysis

All experiments were performed in triplicate. The Student's *t* test was used to determine the statistical significance of the data obtained. The software used was SPSS for window 11.0. $P<0.05$ was taken to represent a statistically significant difference between group means.

## Results

### Construction of shRNAs expressing vectors from pSUPER.retro

We constructed two vectors expressing shRNAs against *bcl-2* and one vector against GL3 gene and named them psB1, psB2 and psGL3 respectively (Fig. 1). DNA sequencing confirmed all vectors were correct.

### Isolation of clones stably expressing shRNA targeting *bcl-2* and GL3 gene

CNE1 cells were transfected with plasmid psB1, psB2 or psGL3 expressing shRNA targeting *bcl-2* or GL3 gene. Several clones were obtained from CNE1 cells transfected with psB1, psB2 or psGL3 after two weeks' puromycin selection. As shown in Fig. 2, Western blotting analysis proved that BCL-2 protein expression was inhibited but not completely in CNE1-psB1 and CNE1-psB2 cells, and not inhibited in CNE1-psGL3 cells compared with CNE1

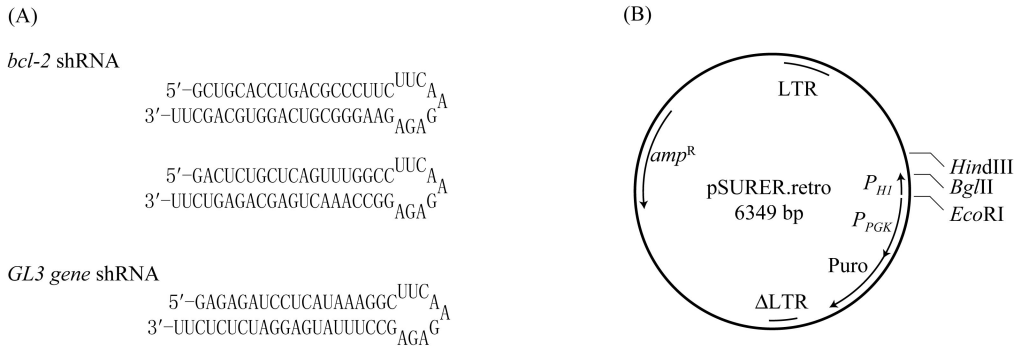


**Fig. 1 (A) Predicted structure of small hairpin RNAs and (B) schematic diagram of the pSUPER.retro vector**

ShRNA encoding template was inserted between the *Hin*dIII and *Bgl*II restriction sites downstream of H1-RNA promoter. $P_{H1}$, H1-RNA promoter; $P_{PGK}$, PGK promoter.

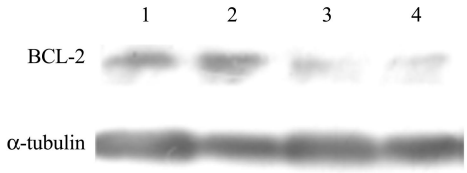


**Fig. 2 The BCL-2 expression in CNE1 cells stably expressing shRNAs against *bcl-2* or GL3 gene**

1, CNE1; 2, CNE1-psGL3; 3, CNE1-psB1; 4, CNE1-psB2.

cells without transfection (Table 1). We selected one positive clone from each group for further analysis. No obvious change in cell morphology was observed in comparison with the parental CNE1 cells.

## Suppression of BCL-2 expression did not affect cell proliferation

The effect of suppressing *bcl-2* on cell growth was determined by MTT assay. As shown in Fig. 3, CNE1, CNE1-psGL3, CNE1-psB1 and CNE1-psB2 cells all exhibited similar proliferation index, suggesting that *bcl-2* gene did not significantly affect cell growth and cell viability. We next used FACScan analysis to examine the profile of cell cycle distribution and apoptosis. Inhibition of BCL-2 did not cause an induction of apoptosis and also had no influence on the cell cycle profile (data not shown).

**Table 1 Inhibition rate of BCL-2 expression in CNE1 cells stably expressing shRNAs against *bcl-2* or GL3 gene**

| Cell line | $\bar{x} \pm s$ [a] | Inhibition rate (%) [b] |
|---|---|---|
| CNE1 | 0.5025 ± 0.0369 | 0 |
| CNE1-psGL3 | 0.5571 ± 0.0324 | −10.87 |
| CNE1-psB1 | 0.0880 ± 0.0053* | 82.49 |
| CNE1-psB2 | 0.0545 ± 0.0062* | 89.15 |

[a] integral absorption ratio (BCL-2/α-tubulin) from Western blotting analysis, experiments were repeated three times; [b] inhibition rate was determined in comparison to untransfected CNE1 cells. *$P$<0.001 vs. CNE1 group.

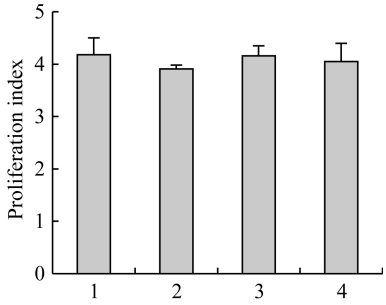


**Fig. 3 The proliferation ability of CNE1 cells stably expressing shRNAs against *bcl-2* or GL3 gene**

1, CNE1; 2, CNE1-psGL3; 3, CNE1-psB1; 4, CNE1-psB2.

## Suppression of BCL-2 induced a significant sensitization of CNE1 cell line to cisplatin

As *bcl-2* gene was identified to play important roles in the resistance to chemotherapy in many tumors, we next investigated if inhibition of *bcl-2* by RNAi affected nasopharyngeal carcinoma cell line's sensitivity to the programmed cell death-inducing drug cisplatin. As shown in Fig. 4, the survival index decreased in CNE1-psB1 and CNE1-psB2 cells with the addition of 4 μg/ml cisplatin

and markedly decreased with 8 μg/ml cisplatin. FACScan analysis confirmed the result. Flow cytometry showed that the rates of cell apoptosis were 11.10%, 15.86%, 22.12% and 48.84%, respectively, when 8 μg/ml cisplatin was added to CNE1, CNE1-psGL3, CNE1-psB1 and CNE1-psB2 cells for 48 h (data not shown).

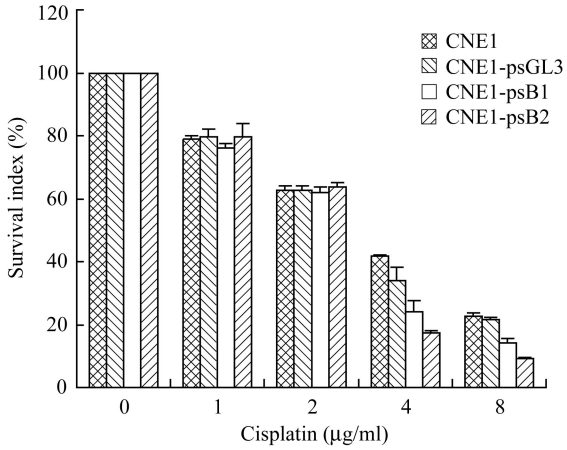


**Fig. 4 The effect of different concentrations of cisplatin on CNE1 cells**

## Discussion

The overexpression of BCL-2 may be present in many malignancies, such as non-Hodgkin's lymphoma, colon cancer, liver cancer and gallbladder cancer, etc.. In about 74.3%–89.0% of the nasopharyngeal carcinoma cases, BCL-2 was overexpressed [16–18]. Sheu *et al.* [19] reported that BCL-2 protein was highly expressed in cancer and atypic hyperplastic epithelium using immunohistochemistry. These suggest that *bcl-2* gene may play an important role in the early stage of nasopharyngeal carcinogenesis.

BCL-2 is an apoptotic inhibitor that may contribute to therapeutic resistance. It was the first oncogene found to function through production of an inhibitor of programmed cell death [20,21]. Apoptotic inhibition by overexpression of BCL-2 or related family members was associated with the increasing resistance to both chemotherapy and radiation [22–25]. *bcl-2* antisense oligonucleotide can increase anticancer drug sensitivity of tumor cells [26–29]. Dr. Jill Lacy reported that *bcl-2* antisense oligonucleotide chemosensitized human nosopharyngeal carcinoma to cisplatin *in vivo* in an SCID xenotransplanatation model (on the 95th Annual Meeting the American Association of Cancer Research, AACR). Anti-BCL-2 ribozyme can also increase cell death by apoptosis [30–32] and sensitize hormone-resistant prostate cancer cells to apoptotic agents [33]. Recently, studies were taken to silence *bcl-2* using RNAi technique. Quantitative analysis performed 48 h after transfection indicated that the protein level of BCL-2 was declined to 18% of the control, and the proliferation of melanoma cells was suppressed to 32%. Moreover, when the total amount of siRNA reached higher than 400 nmol, significant cell apoptosis occurred [34]. Combined the RNAi of *c-raf* and *bcl-2* induced apoptosis in HL-60, U937, and THP-1 cells and increased chemosensitivity to etoposide and daunorubicin [35].

In previous study, we found that NPC cell lines performed poorly in transfection assays. The transient transfection efficiencies were low using GFP and luciferase gene reporter system. Therefore inhibition of *bcl-2* expression could not be distinctly detected by Western blotting analysis after transient transfection (data not shown). We then established three CNE1 cell lines stably expressing shRNAs through puromycin selection. MTT assay and FCM results showed that psB1 and psB2 might not influence cell growth but increase the chemosensiti-vity to cisplatin of NPC cells. For all these reasons, our results regarding the significant biological effects of RNAi directed against the *bcl-2* gene should be considered pro-mising for future RNAi-based therapeutic approaches to NPC or other malignancies, especially considering that RNAi has been repeatedly proven to be more robust than antisense techniques because it works more often, typically decreases expression of a gene to lower level, also acts at concentrations below that required for antisense experiments.

In conclusion, the attempts described herein were directed to explore the function of the RNAi pathway in NPC cells and to evaluate the biological significance of this phenomenon, suggesting the feasibility of NPC cell sensitization to chemotherapy by RNAi for *bcl-2* gene. Further research is necessary to define the optimal therapeutic target genes, and to establish the conditions for *in vivo* and clinical applicability of RNAi.

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