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Bcl-2 Small Interfering RNA Sensitizes Cisplatin-resistant Human Lung Adenocarcinoma A549/DDP Cell to Cisplatin and Diallyl Disulfide

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Abstract Bcl-2 is overexpressed in a variety of human tumors and is involved in tumorigenesis and chemoresistance. In this study, we investigated the inhibitory effect of the hairpin Bcl-2 small interfering (si)RNA on the expression of the *Bcl-2* gene in the cisplatin (DDP)-resistant human lung adenocarcinoma cell line A549/DDP, and the effect of Bcl-2 siRNA on drug sensitization in A549/DDP cells. Bcl-2 siRNA and negative siRNA plasmids were constructed and stably transfected into A549/DDP cells. Reverse transcription-polymerase chain reaction, immunofluorescence microscopy and Western blot analysis were used to detect the target gene expression. Spontaneous cell apoptosis was detected by acridine orange and ethidium bromide staining. Drug sensitivity of the cells to DDP and diallyl disulfide (DADS) was analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. Expression levels of Bcl-2 mRNA and protein in siRNA stable transfectants were clearly reduced compared with negative siRNA transfectants and untreated cells. MTT results indicated that Bcl-2 transfectants had a higher cell inhibition rate after treatment with 0.2–200 μg/ml DDP or 50–200 μM DADS. Flow cytometry revealed increased apoptosis in Bcl-2 siRNA cells. After the addition of 20 μg/ml DDP or 100 μM DADS, siRNA targeting of the *Bcl-2* gene specifically down-regulated gene expression in A549/DDP cells, increased spontaneous apoptosis, and sensitized cells to DDP and DADS.

Keywords small interfering RNA; adenocarcinoma; Bcl-2; A549/DDP; apoptosis

Lung cancer is the leading cause of cancer-related death in the world [1]. Non-small-cell lung cancer (NSCLC) constitutes approximately 80% of all lung cancers, 40% of which are at an advanced stage at the time of diagnosis. Cisplatin (DDP), a commonly used therapeutic agent in NSCLC, together with a third-generation anticancer drug, such as vinorelbine, gemcitabine, or the taxanes, is the standard regimen used in the first-line treatment of advanced NSCLC. Of these regimens, DDP has been evaluated in multiple phase III trials and showed consistent superior efficiency. Diallyl disulfide (DADS), an important component of garlic (*Allium sativum*), has been recently shown to inhibit the growth of human tumor cells from colon, lung, skin, and breast origins. The antiproliferative effect of DADS is due to its ability to suppress the cell division rate and induce apoptosis in human tumor cells. DADS was also reported to induce apoptosis as determined from morphological changes, DNA fragmentation, and the increased proportion of cells in the sub-G_1 population, all of which were observed in cells after exposure. The underlying mechanism involved the up-regulation of apoptotic Bax, and down-regulation of anti-apoptotic Bcl-2 [2–5]. In this study we have down-regulated the expression level of the *Bcl-2* gene by RNA interference in order to determine whether cellular drug sensitivity increased after DADS was combined with Bcl-2 siRNA treatment.

Clinical multidrug resistance to chemotherapeutic agents is a major obstacle to potentially curative treatments for advanced NSCLC [6]. Thus, new methods to improve

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the clinical response to chemotherapy are required. Gene therapy for malignant disease is a promising approach.

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 or the *IAP* and *Mcl-1* families, has been shown to play a critical role [7]. The proto-oncogene *Bcl-2*, discovered in low-grade Burkitt cell lymphomas, is a critical regulator of apoptosis [8]. Among the Bcl-2 protein family members, it has been repeatedly shown that Bcl-2 and Bcl-xl overexpression delays the onset of apoptosis induced by several cytotoxic drugs. Overexpression of Bcl-2 has been associated with several malignancies, including NSCLC [9–12]. Substantial research has shown that downregulation of anti-apoptotic gene expression can sensitize cancer cells to anticancer drugs.

Most chemotherapeutic agents, including DDP and DADS, induce cell apoptosis. Activation of a family of cysteine proteases or caspases is essential for apoptotic cell death [13]. It is believed that DNA damage caused by chemotherapeutic drugs induces the release of mitochondrial cytochrome c, which facilitates activation of initiator caspase-9, thereby triggering activation of downstream effector caspases, such as caspase-3 [14].

Recently, the successful use of small interfering (si) RNA in down-regulating gene expression in several model systems has led to many attempts to explore this methodology in a potentially therapeutic setting [15]. With DDP and DADS as the drugs of choice for NSCLC treatment and the emergence of drug resistance as a critical problem in DDP therapy, we examined the influence of Bcl-2 siRNA on drug sensitization in A549/DDP cells and explored the mechanism of NSCLC cell apoptosis after treatment with DDP and DADS.

Materials and Methods

siRNA vector construction

pSilencer 3.1-H1 linear vector was purchased from Ambion (Austin, USA). The Bcl-2 siRNA insert sequence was equivalent to GenBank accession No. Z23115, with sense (5′-AGTACATCCATTATAAGCT-3′) and antisense (5′-AGCTTATAATGGATGTACT-3′) sequences. A negative control vector that expresses a hairpin siRNA with limited homology to any known sequences of the human genome was commercially available (Ambion). Plasmid DNA was purified by cesium chloride bromide gradient centrifugation. The purified DNA was diluted to 1 mg/ml and stored at −20 ºC until used.

Cell culture and transfection

The human lung adenocarcinoma cell line A549 and the DDP-resistant cell line A549/DDP were purchased from the Xiangya Cell Center, Central South China University (Changsha, China). The cells were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% bovine calf serum (Hyclone, Logan, USA), and, for A549/DDP, 2 μg/ml DDP (Sigma-Aldrich, St. Louis, USA) was added. Twelve hours before transfection, cells were seeded into wells of a 24-well plate that contained antibiotic-free medium; at the time of transfection, the cell confluence was routinely 90%–95%. Transfection was carried out according to the manufacturer's protocol. Bcl-2 siRNA or negative siRNA plasmid (0.8 μl) was diluted with 50 μl OPTI-MEM (Invitrogen) or 2 μl Lipofectamine 2000 (Invitrogen) with 50 μl OPTI-MEM. After 5 min, the dilutions were mixed together and incubated at 37 ºC for 25 min, then dispensed into each well. Forty-eight hours after transfection, 700 μg/ml G418 (Amresco, Solon, USA) was added to the medium to select transfected Bcl-2 siRNA and negative siRNA cells. Three to five cell clones with resistance to G418 were picked and added to culture medium containing 300 μg/ml G418.

Reverse transcription-polymerase chain reaction (RT-PCR)

Transfected and untreated cells were collected and washed with phosphate-buffered saline (PBS). Total RNA were extracted from the cells using a total RNA isolation kit (Bio Basic, Markham, Canada) according to the manufacturer's protocol. Three micrograms of total RNA were used for RT-PCR with a total volume of 20 μl with the Superscript preamplification system (Promega, Madison, USA). Aliquots of cDNA (3 μl) were amplified in a total volume of 50 μl using the GeneAmp PCR kit (Promega) following the conditions recommended by the manufacturer. The sense and antisense primers for Bcl-2 were 5′-TGGATGTTCTGTGCCTGTAAAC-3′ and 5′-TGATGCGGAAGTCACCGAAA-3′(amplification product 571 bp), respectively. The cycling conditions were 94 ºC for 4 min, followed by 35 cycles of 94 ºC for 30 s, 52 ºC for 45 s, and 72 ºC for 30 s, with a final extension of 72 ºC for 10 min. The sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase were 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′(amplification

product 306 bp), respectively. The cycling conditions were 95 ºC for 5 min, followed by 35 cycles of 95 ºC for 1 min, 55 ºC for 1 min, and 72 ºC for 1 min, with a final extension of 72 ºC for 10 min. PCR products were separated on a 1.3% agarose gel and viewed by ethidium bromide (EB; Molecular Probes, Eugene, USA) staining. The data were analyzed using AlphaImager 2200 software (Alpha Innotech, San Leandro, USA).

Immunofluorescence microscopy

A549/DDP cells were collected, washed twice with PBS, and fixed with methanol:acetic acid at a 3:1 dilution for 15 min at room temperature. The cells were permeabilized with PBS containing 0.25% Triton X-100 and 5% dimethylsulfoxide (Sigma-Aldrich) for 30 min at 37 ºC and washed twice with PBS. Then the cells were incubated with the anti Bcl-2 primary antibody (Santa Cruz Biotechnology, Santa Cruz, USA), at a dilution of 1:100 in PBS, for 60 min at 37 ºC. After three washes, the cells were incubated with the goat anti-rabbit fluorescein-isothiocyanate-junctured secondary antibody (Santa Cruz Biotechnology) for 60 min at 37 ºC and washed three times with PBS, then analyzed by fluorescence microscopy (Olympus, Tokyo, Japan) using the 20× objective. Data were acquired with a Pixera camera (Pixera, Los Gatos, USA).

Western blot analysis

Cells were lyzed in a lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mg/ml aprotinin, 100 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and PBS (pH 7.4). Cell lysates were centrifuged at 10,000 *g* for 10 min at 4 ºC, and the protein content in the supernatants was determined using a BCA protein assay kit (Pierce, Rockford, USA). Equal amounts of protein lysate were electrophoretically separated on 10% or 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, USA). After blocking, each membrane was incubated with rabbit anti-Bcl-2 monoclonal antibody and goat anti-caspase-3 polyclonal antibody (Santa Cruz Biotechnology), or rabbit anti-poly(ADP-ribose) polymerase (PARP) polyclonal antibody (Cell Signaling, Beverly, USA) overnight at 4 ºC and further incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-goat or anti-rabbit secondary antibody (Santa Cruz Biotechnology). Bound antibodies were detected by an enhanced chemiluminescence kit (Santa Cruz Biotechnology) using a Lumino image analyzer (Taitec, Tokyo, Japan).

Apoptosis analysis

Cell apoptosis was identified by fluorescence staining with acridine orange (AO; Becton Dickinson, Franklin Lakes, USA) and EB. For the morphological examination of apoptosis, cells were seeded in a 24-well microplate and washed with PBS, mixed with the same volume of a dual AO/EB solution consisting of both compounds of 100 μg/ml. The final volume (200 μl) was observed using a fluorescence microscope at an objective magnification of 20× (Olympus). For quantification, three different fields were counted and at least 300 cells were enumerated in each field. All experiments were done in triplicate.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were incubated for 24 h in a 96-well microplate with various concentrations of DDP and DADS. After 48 h, 5 mg/ml MTT was added and the cells were further incubated at 37 ºC for 4 h. Dimethylsulfoxide (200 μl) was added into each well and incubated for 10 min. The reaction was optically monitored at 570 nm (A_{570}) using a 96-well microtiter plate reader (Pharmacia, Piscataway, USA). All experiments were carried out in triplicate. The inhibitory rate of A549/DDP cells was calculated according to **Equation 1**:

$$IR=[A_{570}(\text{control})-A_{570}(\text{drug})]/A_{570}(\text{control})\times 100\% \quad (1)$$

where A_{570} (control) was the absorbance in Bcl-2 siRNA or negative siRNA or control groups, and A_{570} (drug) was the absorbance in the drug-treated group.

Flow cytometry

All cells were treated with 20 μg/ml DDP or 100 μM DADS, washed twice in PBS, and fixed with 70% ethanol overnight at 4 ºC. The cells were then washed once with PBS and stained with 800 μl of 50 μg/ml propidium iodide (Sigma-Aldrich) at room temperature for 30 min. The cell apoptosis was determined by flow cytometry (Beckman Coulter, Fullerton, USA) and analyzed with CellQuest software version 3.3 (Becton Dickinson, San Jose, USA).

Statistical analysis

Statistical analysis was carried out using SPSS software (version 11.0; SPSS, Chicago, USA). Data were expressed as the mean±SD and analyzed by one-way ANOVA and the least significant difference tests. $P<0.05$ was considered significant.

Results

Overexpression of Bcl-2 in DDP-resistant cells

To understand the underlying mechanisms of DDP resistance, RT-PCR and Western blot analysis were used to evaluate Bcl-2 expression. The results revealed elevated mRNA and protein levels in A549/DDP cells compared to A549 cells (**Fig. 1**).

Inhibition of Bcl-2 mRNA expression by siRNA

A549/DDP cells were transfected with Lipofectamine 2000 *in vitro*. After 48 h, G418 was added to select transfected Bcl-2 siRNA and negative siRNA cells. Cell clones were formed after 14 d of further incubation, and Bcl-2 mRNA levels were then determined in A549/DDP cells transfected with Bcl-2 siRNA by RT-PCR. The Bcl-2 transcript of transfected A549/DDP cells was significantly less than that of the negative siRNA or untreated control groups (**Fig. 2**).

Bcl-2 siRNA efficiently inhibited Bcl-2 protein expression and activities of caspase-3 and PARP

To further confirm whether the Bcl-2 protein level was also decreased by Bcl-2 SiRNA, we measured the Bcl-2 protein expression in A549/DDP cells by immunofluorescence microscopy and Western blotting. As shown in **Fig. 3**, the Bcl-2 protein was expressed at a higher level than in normal cells and cells transfected with negative vector than cells transfected with Bcl-2 siRNA vector. Caspase-3 and PARP activities were found increased in cells transfected with Bcl-2 siRNA compared with cells transfected with the vector control (**Fig. 4**).

Spontaneous apoptosis induced by Bcl-2 siRNA

To investigate the effect of siRNA-induced Bcl-2 down-regulation on cell apoptosis, Bcl-2 siRNA or negative siRNA stably transfected cells were collected and stained with AO/EB. The results showed that the cells with Bcl-2 siRNA underwent typical apoptotic morphological changes of nuclear and cytoplasmic condensation, loss of cell volume,

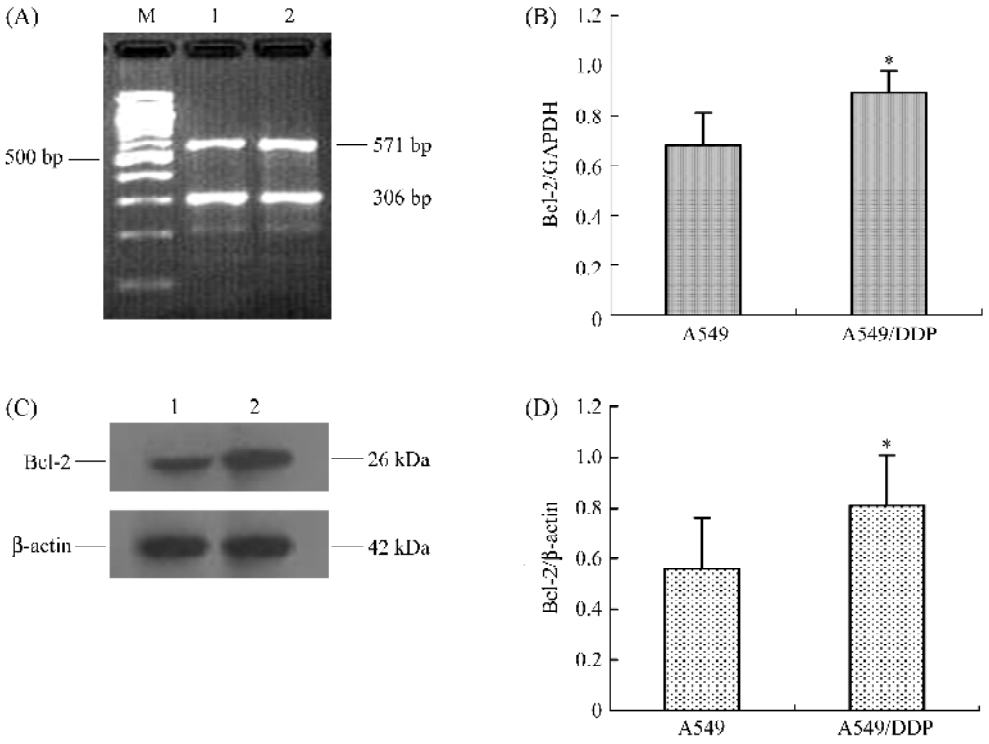

Fig. 1 Expression of Bcl-2 in human lung adenocarcinoma cell line A549 and cisplatin (DDP)-resistant cell line A549/DDP

(A) Reverse transcription–polymerase chain reaction was used to detect Bcl-2 mRNA expression in A549 (lane 1) and A549/DDP (lane 2) cells. M, DNA ladder. (B) Bcl-2 mRNA expression was increased in A549/DDP cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C) Western blot analysis was used to detect Bcl-2 protein expression in A549 (lane 1) and A549/DDP (lane 2) cells. β-Actin was used as the control. (D) Bcl-2 protein expression was increased in A549/DDP cells. The results shown are representative of three independent experiments. The histogram shows the mean±SD. * $P<0.05$ versus A549 cells.

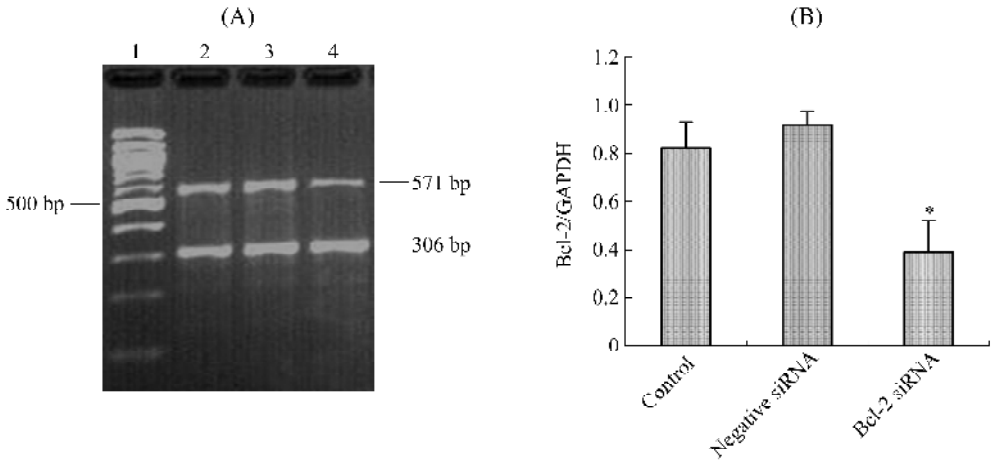

Fig. 2 Effects of Bcl-2 small interfering (si)RNA on Bcl-2 mRNA

(A) Reverse transcription-polymerase chain reaction was used to detect Bcl-2 mRNA expression. Human lung adenocarcinoma cell line A549 and cisplatin (DDP)-resistant cell line A549/DDP was used. 1, 100 bp DNA Ladder; 2, untreated control; 3, negative siRNA transfectants; 4, Bcl-2 siRNA transfectants. Polymerase chain reaction product length: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 306 bp; Bcl-2, 571 bp. (B) Bcl-2 mRNA expression was significantly decreased in Bcl-2 siRNA transfected cells. The results shown are representative of three independent experiments. The histogram shows the mean±SD. *P<0.05 versus negative siRNA or control group.

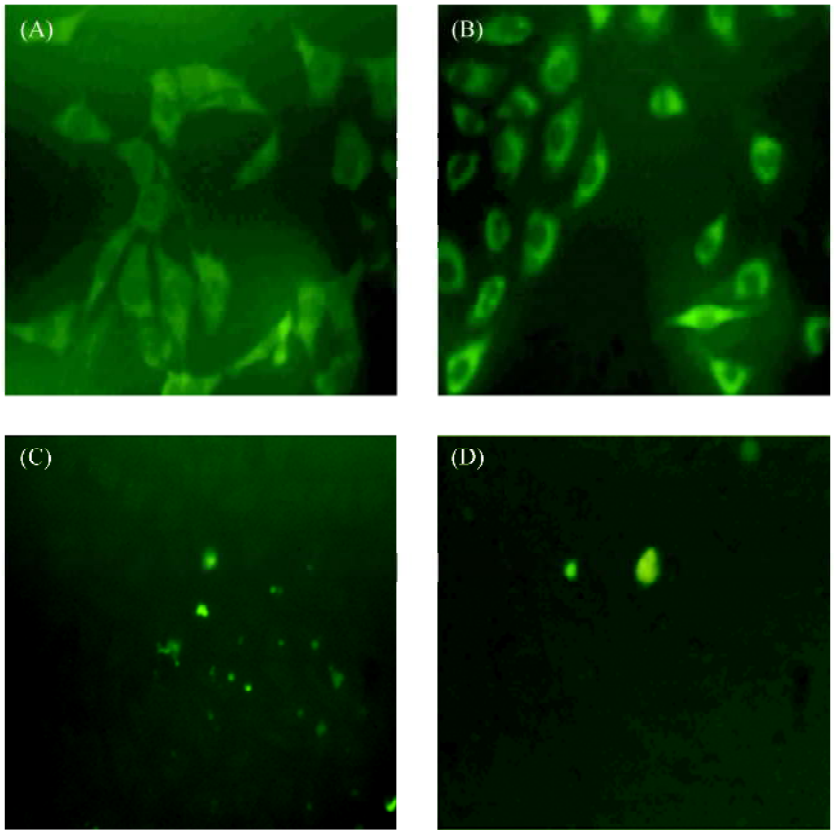

Fig. 3 Effects of Bcl-2 small interfering (si)RNA on Bcl-2 protein expression human lung adenocarcinoma cells

Immufluorescence was used to detect Bcl-2 protein expression. (A) Untreated control. (B) Negative siRNA transfectants. (C) Bcl-2 siRNA transfectants. (D) Secondary antibody only. We detected very low fluorescence in (C) similar to (D). The results shown are representative of three independent experiments. Magnification, 200×.

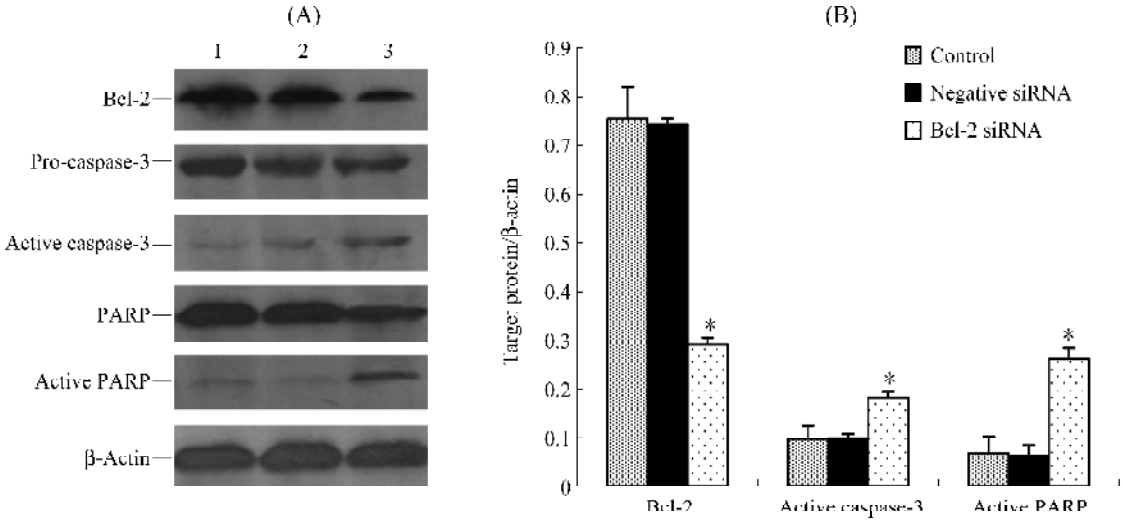

Fig. 4 Effects of Bcl-2 small interfering (si)RNA on Bcl-2, caspase-3 and poly(ADP-ribose) polymerase (PARP) protein expression in human lung adenocarcinoma cells

(A) Western blotting was used to detect Bcl-2, caspase-3, and PARP protein expression. The experiments were carried out in triplicate and representative data are shown. 1, untreated control; 2, negative siRNA transfectants; 3, Bcl-2 siRNA transfectants. Bcl-2 protein expression was significantly reduced in Bcl-2 siRNA transfected cells. Active caspase-3 and active PARP protein expression was increased in Bcl-2 siRNA transfected cells. The expression of β-actin protein was not changed. (B) Relative protein expression level of Bcl-2, active caspase-3, and active PARP compared with β-actin. **P*<0.05 versus negative siRNA or untreated control group.

and nuclear fragmentation. In contrast, untreated control cells and negative siRNA transfected cells did not show these apoptotic characteristics (**Fig. 5**).

Influence of Bcl-2 down-regulation on cell susceptibility to DDP and DADS-induced death

MTT assay results showed that Bcl-2 siRNA transfectants had a lower cell viability and higher inhibition rate than that of the negative vector or untreated control cells after treatment with various concentrations of DDP and DADS: 0.2 μg/ml DDP (3.31%±0.78% versus 1.14%±0.98% and 1.72%±1.02%, respectively); 2 μg/ml DDP (6.52%±0.76% versus 1.34%±0.89% and 2.70%±0.66%, respectively); 20 μg/ml DDP (46.19%±3.90% versus 26.96%±6.08% and 27.26±4.12%, respectively); 200 μg/ml DDP (50.51%±9.67% versus 33.88%±5.90% and

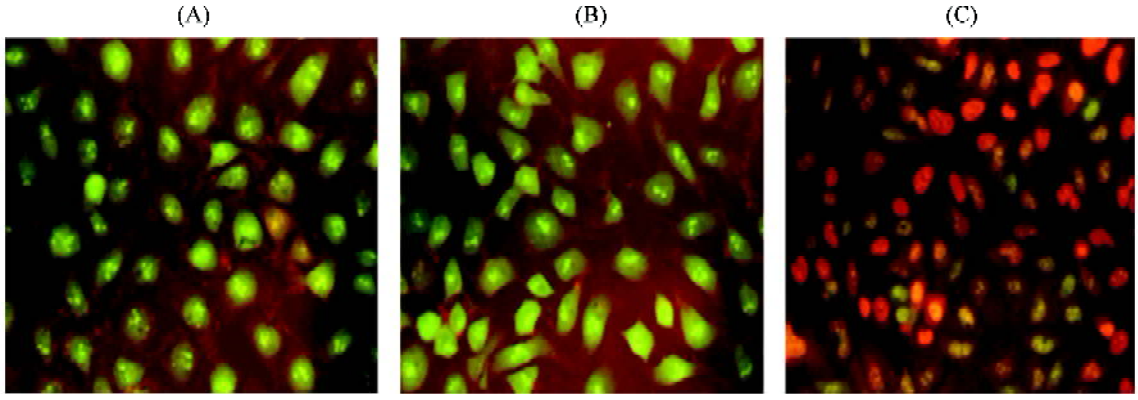

Fig. 5 Spontaneous apoptosis of cisplatin-resistant human lung adenocarcinoma cells induced by small interfering (si)RNA (acridine orange/ethidium bromide staining)

The spontaneous apoptosis was observed by immunofluorescence. The experiments were carried out in triplicate, and representative datas are shown. (A) Untreated control. (B) Negative siRNA transfectants. (C) Bcl-2 siRNA transfectants. Magnification, 200×.

33.27%±3.58%, respectively); 50 μM DADS (4.46%± 0.97% versus 2.16%±0.86% and 2.95%±1.03%, respectively); 100 μM DADS (17.19%±7.01% versus 11.93%±4.90% and 10.21±5.65%, respectively); 150 μM DADS (25.75%±7.09% versus 20.86%±10.12% and 19.45%±6.78%, respectively); and 200 μM DADS (35.21%±7.90% versus 25.53%±8.57% and 25.81%± 11.45%, respectively) (**Table 1**). The inhibition rate showed a dose-dependent effect of DDP and DADS. Moreover, flow cytometry showed that cells with Bcl-2 siRNA had a markedly increased apoptosis population compared with negative siRNA or untreated control cells after the addition of 20 μg/ml DDP for 48 h and 100 μM DADS for 24 h (**Fig. 6**).

Table 1 Inhibition rate (IR) of Bcl-2 small interfering (si)RNA transfected cells, negative siRNA transfected cells and normal cells after treatment with cisplatin (DDP) and diallyl disulfide (DADS)

Group	IR (%)							
	DDP (μg/ml)				DADS (μM)			
	0.2	2	20	200	50	100	150	200
Bcl-2 siRNA	3.31*	6.52*	46.19*	50.51*	4.46*	17.19*	25.75	35.21*
Negative siRNA	1.14	1.34	26.96	33.88	2.16	11.93	20.86*	25.53
Control	1.72	2.70	27.26	33.27	2.95	10.21	19.45	25.81

ANOVA analysis shows the mean difference of the Bcl-2 siRNA transfected group is significant at the concentration of 0.2, 2, 20, and 200 μg/ml of DDP or 50, 100, 150, 200 μM DADS. * P<0.05 versus negative or control group.

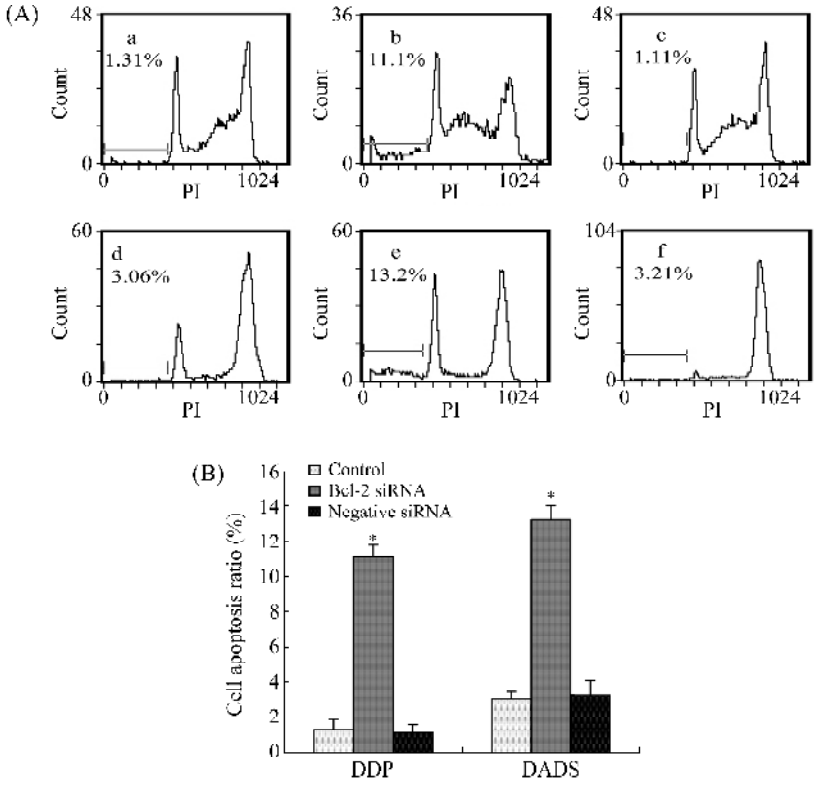

Fig. 6 Flow cytometry analysis of human lung adenocarcinoma cells after treatment with 20 μg/ml cisplatin (DDP) and 100 μM diallyl disulfide (DADS) for 48 h

(A) Flow cytometry analysis of propidium iodide-stained cells was carried out, with the percentages of apoptosis cells shown. The experiments were carried out in triplicate and representative data are shown. a, untreated control+DDP; b, Bcl-2 small interfering (si)RNA transfectants+DDP; c, negative siRNA transfectants+DDP; d, untreated control+DADS; e, Bcl-2 siRNA transfectants+DADS; f, negative siRNA transfectants+DADS. (B) Data represent the mean±SD of cell apoptosis ratio from three determinations. ANOVA analysis was used. *P<0.005 versus negative siRNA or untreated control group.

Discussion

Bcl-2 is widely used as a target for cancer chemotherapeutics. Antisense and small molecule inhibitors of Bcl-2 are being developed to down-regulate Bcl-2 to enhance anticancer drug sensitivity or to reverse drug resistance [16,17].

In this study, we found that Bcl-2 mRNA and protein expression was increased in the DDP-resistant A549/DDP cell line, and the expression level was obviously higher than in the A549 cell line. Suppression by Bcl-2-specific siRNA led to a greater decrease in the mRNA and protein expression of Bcl-2 and growth inhibition in DDP-resistant cells. We also found that the apoptosis was increased in Bcl-2 siRNA transfectants.

DDP and DADS induce cell death by apoptosis. Activation of a family of cysteine proteases or caspases is essential for cell death by apoptosis [11]. The activation of executioner caspases results in the cleavage of critical cellular proteins, such as PARP, DNA-dependent protein kinase, lamin B, and protein kinase Cy. Apoptosis is regulated by a complex cellular signaling network and a defect in apoptotic signaling can contribute to drug resistance.

Our results showed Bcl-2 siRNA can decrease Bcl-2 protein expression and activate procaspase-3, followed by the cleavage of their substrate PARP, suggesting that Bcl-2 siRNA induces cytochrome c-mediated caspase-dependent apoptosis in human NSCLC cells. Broader caspase inhibitors are being used to confirm this result in our laboratory.

The development of RNA interference technology has made it possible to suppress the function of specific molecular targets. This technology will be very useful in developing new treatments for cancer [18–25] because our knowledge of molecular targets that demarcate the difference between normal and malignant cells is increasing. Our study results on A549/DDP suggest Bcl-2 protein as a good target for cancer therapy, especially in cancers resistant to conventional chemotherapy. Nevertheless, *in vivo* delivery and tumor specificity are challenging issues for the use of Bcl-2 siRNA as an anticancer therapeutic agent [26]. Development of genetic vectors or formulations for *in vivo* delivery of siRNA will be necessary before siRNA can be used as a therapeutic agent.

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