Bcl-XL Small Interfering RNA Sensitizes Cisplatin-resistant Human Lung Adenocarcinoma Cells

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Abstract

Bcl-XL is overexpressed in a variety of human tumors and is involved in tumorigenesis and chemoresistance. This study investigated the inhibitory effect of the hairpin Bcl-XL small interfering RNA (siRNA) on the expression of the Bcl-XL gene in the cisplatin (DDP)-resistant human lung adenocarcinoma cell line A549/DDP, and the effect of Bcl-XL siRNA on drug sensitization in A549/DDP cells. Bcl-XL siRNA and negative siRNA plasmids were constructed and stably transfected into A549/DDP cells. Reverse transcription-polymerase chain reaction and Western blot analysis were used to detect the target gene expression. Spontaneous apoptosis of cells was detected by acridine orange and ethidium bromide staining. Drug sensitivity of the cells to DDP was analyzed with dimethylthiazol-diphenyltetrazolium bromide (MTT) and flow cytometry. Expression levels of Bcl-XL mRNA and protein in siRNA stable transfectants were clearly reduced as compared with negative siRNA transfectants and untreated cells. MTT results indicated that Bcl-XL transfectants had a higher cell inhibition rate than the negative vector or untreated cells after treatment with 0.2–200 μg/ml DDP. Flow cytometry revealed increased apoptosis in Bcl-XL siRNA cells. After the addition of 20 μg/ml DDP, siRNA targeting of the Bcl-XL gene specifically down-regulated gene expression in A549/DDP cells, increased spontaneous apoptosis, and sensitized cells to DDP. The results showed that Bcl-XL siRNA contributed to an increase of DDP-induced cell death in non-small-cell lung cancer and sensitized cells to DDP, leading to increased the effectiveness of the drug in treating non-small-cell lung cancer.

Key words  apoptosis; small interfering RNA; adenocarcinoma; Bcl-XL; A549/DDP

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, with 40% being at an advanced stage at the time of diagnosis. NSCLC represents the most frequent and therapy-refractory subclass of lung cancer. Improving apoptosis induction in NSCLC represents a logical way forward in treating this tumor. Cisplatin (DDP), a commonly used therapeutic agent in NSCLC, plus 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 shown consistent superior efficacy. Systemic chemotherapy is warranted, but is hampered by the chemoresistance of most NSCLCs [2]. Clinical multidrug resistance to chemotherapeutic agents is a major obstacle to potentially curative treatments for advanced NSCLCs. Thus, new methods to improve the clinical response to chemotherapy are required. Gene therapy for malignant disease is a promising approach.

The proto-oncogene Bcl-2, first discovered in low-grade Burkitt cell lymphomas, is a critical regulator of apoptosis [3]. Bcl-2 represses cell death triggered by a diverse array of stimuli including chemotherapy and γ-irradiation. Bcl-XL is a functional and structural homolog of Bcl-2, which also provides protection against a wide variety of chemotherapeutic agents [4]. Overexpression of Bcl-XL is linked with the occurrence of malignancies including NSCLC [5]. A previous study showed that the expression of Bcl-XL is higher in DDP-resistant NSCLC cells than in...
normal NSCLC cells [6]. Interestingly, the expression level of the Bcl-2 family proteins changes as tumors become more malignant or after treatment, suggesting that expression of these survival proteins is critical not only for tumor development but also for tumor progression and resistance to therapy [7]. Most chemotherapeutic agents including DDP induce cell apoptosis. Activation of a family of cysteine proteases or caspases is essential for apoptotic cell death [8]. 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 [9]. Apoptosis is regulated by a complex cellular signaling network and a defect in apoptotic signaling can contribute to drug resistance.

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 [10]. As DDP is the drug choice for the treatment of NSCLC and the emergence of DDP resistance is a critical problem in DDP therapy, we decided to examine the influence of Bcl-XL siRNA on drug sensitization in A549/DDP cells and explore the mechanism of NSCLC cell apoptosis after treatment with DDP.

Materials and Methods

siRNA vector construction

pSilencer 3.1-H1 linear vector was purchased from Ambion (Austin, USA). The Bcl-XL siRNA insert sequence is equivalent to GenBank accession No. Z23115. The Bcl-XL siRNA insert sequence had sense (5'-CAGGG-ACAGCATATCAGAG-3') and antisense (5'-GTCCCCGTGTGATAGTCTC-3') sequences. A negative control vector that expresses a hairpin siRNA with limited homology to any known sequences of 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 °C 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, South China University (Changsha, China). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ 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). 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 monolayers were routinely 90%–95% confluent. Transfection was carried out according to the manufacturer’s protocol. Bcl-XL 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 °C 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-XL siRNA and negative siRNA cells. Three to five cell clones formed in each microplate during the 14 d incubation were picked and added to culture medium containing 300 μg/ml G418. Bcl-XL mRNA was assessed in A549/DDP cells transfected with Bcl-XL siRNA.

Reverse transcription-polymerase chain reaction (RT-PCR)

Transfected and untreated cells were collected and washed with phosphate-buffered saline (PBS). Total RNA was 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 was used for RT in a total volume of 20 μl with the Super-script 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-XL gene were 5'-CTGGGTGTGACTTCTCCTCA-3' and 5'-GCATC-TCTTGTCTACGCTTT-3' (amplification product 457 bp), respectively. The cycling conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 60 s, and a final extension of 72 °C for 10 min. The sense and antisense primers for GAPDH were 5'-TCACCATTTCCAGGAGCCAG-3' and 5'-TGTC-GCTGTTGAAGTCAGAG-3' (amplification product 648 bp), respectively. The cycling conditions were 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, and a final extension of 72 °C for 10 min. PCR products were separated on a 1.3% agarose gel and viewed by ethidium bromide (EB) staining. The data were analyzed using Alphalmager 2200 software (Alpha Innotech, San Leandro, USA).
Western blotting

Cells were homogenized 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 lysate protein 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 mouse anti-Bcl-XL monoclonal antibody and goat anti-caspase-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA), 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 ECL 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) and EB (Molecular Probes, Eugene, USA). 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 100 μg/ml of both compounds. The final volume (200 μl) was observed using a fluorescence microscope with an objective magnification of 20 times (Olympus, Tokyo, Japan). For quantification, three different fields were counted and at least 300 cells were enumerated in each field. All experiments were repeated in triplicate.

Dimethylthiazol-diphenyltetrazolium bromide (MTT) assay

Cells were incubated for 24 h in a 96-well microplate with various concentrations of DDP. After 48 h, 5 mg/ml MTT was added and the cells were further incubated at 37 ºC for 4 h. Two hundred microliters of dimethylsulfoxide was added into each well and incubated for 10 min. The reaction was monitored optically at 570 nm (A570) 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:

\[ \text{IR} = \frac{A_{570} \text{(control)} - A_{570} \text{(drug)}}{A_{570} \text{(control)}} \times 100\% \]

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

Flow cytometry

All cells were treated with 20 μg/ml DDP, washed twice in PBS, and fixed with 70% ethanol overnight at 4 °C. 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 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 ANOVA and the least significant difference test. \(P<0.05\) was considered significant.

Results

Overexpression of Bcl-XL in DDP-resistant cells

To evaluate the underlying mechanisms of resistance to DDP, Western blot analysis was used to evaluate Bcl-XL expression. The results revealed an elevated protein level in A549/DDP cells compared to A549 cells. (Fig. 1)

Inhibition of Bcl-XL mRNA expression by Bcl-XL siRNA

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

Bcl-XL siRNA influence on Bcl-XL protein expression and activities of caspase-3 and PARP

To examine whether the Bcl-XL protein was also silenced, its expression was measured by Western blotting.
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Fig. 1  Expression of Bcl-XL in human lung adenocarcinoma cell line A549 and cisplatin (DDP)-resistant cell line A549/DDP
(A) Western blotting detected Bcl-XL protein expression. 1, A549; 2, A549/DDP.
(B) Bcl-XL protein expression was increased in A549/DDP. *P<0.05 vs. A549

Fig. 2  Effects of Bcl-XL small interfering (si)RNA on Bcl-XL mRNA
(A) Reverse transcription-polymerase chain reaction detected Bcl-XL mRNA expression. 1, 100 bp DNA ladder; 2, control; 3, Bcl-XL siRNA transfectants; 4, negative siRNA transfectants. Polymerase chain reaction product length: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 648 bp; Bcl-XL, 457 bp. (B) Bcl-XL mRNA expression was significantly reduced at Bcl-XL siRNA transfected cells. *P<0.05 vs. negative or normal group.

The negative vector had no significant effect on Bcl-XL expression. Bcl-XL protein was expressed at a higher level in normal cells and cells transfected with negative vector than in the cells transfected with Bcl-XL siRNA vector. Moreover, caspase-3 and PARP activities were clearly increased in cells transfected with Bcl-XL siRNA (Fig. 3).

Fig. 3  Effects of Bcl-XL small interfering (si)RNA on Bcl-XL, caspase-3 and poly(ADP-ribose) polymerase (PARP) protein expression
Western blot detected Bcl-XL, caspase-3 and PARP proteins expression. (A) Bcl-XL protein: 1, control; 2, negative siRNA; 3, Bcl-XL siRNA transfected cells. Bcl-XL protein expression was significantly reduced at Bcl-XL siRNA transfected cells. (B) Caspase-3 protein: 1, control; 2, negative siRNA; 3, Bcl-XL siRNA transfected cells. Active caspase-3 protein expression was increased. (C) PARP protein: 1, control; 2, negative siRNA; 3, Bcl-XL siRNA active. PARP protein expression was up-regulated when Bcl-XL proteins were down-regulated. (D) β-actin protein: 1, control; 2, negative siRNA; 3, Bcl-XL siRNA transfected cells. (E) Bcl-XL, active caspase-3 and active PARP/β-actin ratio. *P<0.05 vs. negative or normal group.
Spontaneous apoptosis induced by siRNA

Having shown that Bcl-XL siRNA could down-regulate mRNA and protein expression of Bcl-XL, we sought to ascertain whether transfected Bcl-XL siRNA cells became more spontaneously apoptotic. Cell morphology was investigated. Bcl-XL siRNA or negative siRNA stably transfected cells were collected and stained with AO/EB. The results showed that the Bcl-XL siRNA transfectants underwent typical apoptotic morphological changes of nuclear and cytoplasmic condensation, loss of cell volume, and nuclear fragmentation. In contrast, untreated cells and negative siRNA transfected cells did not show these apoptotic characteristics (Fig. 4).

Fig. 4 Spontaneous apoptosis of human lung adenocarcinoma cells induced by small interfering (si)RNA (acridine orange/ethidium bromide staining)

The spontaneous apoptosis was observed by immunofluorescence. (A) Control. (B) Negative siRNA transfectants. (C) Bcl-XL siRNA transfectants. Magnification, 20×.

Influence of Bcl-XL down-regulation on cell susceptibility to DDP-induced death

MTT assay results showed that Bcl-XL transfectants had a lower cell viability and higher inhibition rate than that of negative vector or untreated cells after treatment with various concentrations of DDP: 0.2 µg/ml (12.65±1.70% vs. 2.18±0.65% and 2.16±0.80%), 2 µg/ml (49.24±0.48% vs. 4.74±0.65% and 7.47±0.85%), and 200 µg/ml (58.75±0.45% vs. 21.69±0.79% and 18.27±1.23%), DDP (P<0.05 vs. negative siRNA or untreated cells) (Fig. 5). The inhibition rate showed a dose-dependent effect of DDP. Moreover, flow cytometry showed that Bcl-XL siRNA cells had a markedly increased sub-G1 population compared with negative siRNA or untreated cells after the addition of 20 µg/ml DDP for 48 h (Fig. 6).

Fig. 5 Inhibition of Bcl-XL small interfering (si)RNA on cisplatin (DDP)-resistant human lung adenocarcinoma cell line A549/DDP

ANOVA analysis shows the mean difference of the Bcl-XL siRNA transfected group is significant at the concentration of 0.2, 2, 20, and 200 µg/ml of DDP. *P<0.05 vs. negative or normal group.

Discussion

In this study, we show that Bcl-XL mRNA and protein expression decreases in the DDP-resistant A549/DDP cell line. Bcl-XL protein is known to promote cell survival by regulating the electrical and osmotic homeostasis of mitochondria in response to various stimuli [7,11], and down-regulation of Bcl-XL protein by chemicals, antisense oligonucleotides, or siRNA can induce subsequent mitochondrial-based cell death [12–16]. Presently, suppression by Bcl-XL-specific siRNA led to a greater decrease in the mRNA and protein expression of Bcl-XL and growth inhibition in DDP-resistant cells. We also found that the apoptosis was increased in Bcl-XL siRNA transfectants.

Caspases play important roles in apoptosis triggered by various proapoptotic signals [17,18]. In general, activation of the caspase cascade requires both initiator caspases
such as caspase-8, -9, and -10, and effector caspases such as caspase-3, -6, and -7. The effector caspases cleave several vital substrates such as PARP and DFF45, leading to apoptosis. It has been well documented that cytochrome c release from mitochondria and its activation of caspase-9 through binding to the protein Apaf-1 mediates apoptosis triggered by signals such as chemotherapeutic agents [19]. Presently, Bcl-XL siRNA triggered a decrease of Bcl-XL protein expression and the activation of procaspase-3, followed by the cleavage of PARP. We suggest that Bcl-XL siRNA induces cytochrome c-mediated caspase-dependent apoptosis in human NSCLC cells. To confirm this suggestion, planned experiments will use a broad caspase inhibitor.

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 [20–22] because our knowledge of molecular targets that demarcate the difference between normal and malignant cells is increasing. Our study results on human lung adenocarcinoma cells resistant to DDP suggest that Bcl-XL protein is a good target for cancer therapy, especially for cancers resistant to conventional chemotherapy. Nevertheless, in vivo delivery and tumor specificity are challenging issues for the use of Bcl-XL siRNA as an anticancer therapeutic agent [23]. Development of genetic vectors or formulations for in vivo delivery of siRNA will be necessary before siRNA can be used as a therapeutic agent.

**References**


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