Pro-apoptotic effects of splice-switching oligonucleotides targeting Bcl-x pre-mRNA in human glioma cell lines

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Abstract. Alternative splicing is a near-ubiquitous phenomenon with important roles in human diseases, including cancers. Splice-switching oligonucleotides (SSOs) have emerged as a class of antisense therapeutics that modulate alternative splicing by hybridizing to the pre-mRNA splice site. The Bcl-x gene is alternatively spliced to express anti-apoptotic Bcl-xL and pro-apoptotic Bcl-xS. Bcl-xL expression is upregulated in many cancers and is considered a general mechanism by which cancer cells evade apoptosis. By redirecting Bcl-x pre-mRNA splicing from Bcl-xL to Bcl-xS, SSO exerted pro-apoptotic and chemosensitizing effects in various cancer cell lines. In this study, we investigated the effects of SSO targeting Bcl-x pre-mRNA in human glioma cell lines. First, we performed reverse transcription-polymerase chain reaction (RT-PCR) and western blotting to determine the mRNA and protein expression levels of Bcl-xL in glioma cell lines (U87 and U251) and a normal human astrocyte cell line (HA1800). Then, the Bcl-x SSO was designed to bind to the downstream 5' alternative splice site of exon 2 in Bcl-x pre-mRNA and was modified using 2'-O-methoxyethyl-phosphorothioate. An oligonucleotide targeting aberrantly spliced human β-globin intron was used as a negative control. The SSOs were delivered with a cationic lipid into glioma and astrocyte cell lines. The antitumor effects of the SSOs were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and flow cytometry, and the switch in production from Bcl-xL to Bcl-xS was analyzed by RT-PCR and western blotting. Bcl-xL mRNA and protein were highly expressed in both glioma cell lines. The Bcl-x SSO modified Bcl-x pre-mRNA splicing and had pro-apoptotic effects on the glioma cell lines. By contrast, the lipid alone and the control SSO did not affect Bcl-xL expression or induce apoptosis. Our study demonstrated the antitumor activity of an SSO that targets Bcl-x pre-mRNA splicing in glioma cell lines. Bcl-x SSO may be a potential strategy for treating gliomas.

Introduction

Alternative splicing is the process by which splice sites in precursor messenger RNA (pre-mRNA) are differentially selected and paired to produce multiple mature mRNAs and protein isoforms with distinct structural and functional properties. Alternative splicing is a very accurate, efficient, and extraordinarily flexible process that regulates many major aspects of eukaryotic cell biology. Approximately 95% of human genes with multiple exons undergo alternative splicing during pre-mRNA maturation (1-3). In addition to its role in human proteome diversity, alternative splicing is now accepted to play important roles in human diseases, including diabetes, neurodegenerative diseases, and cancer (4-6).

Aberrant alternative splicing has two major roles in cancer by promoting the emergence of a cancer-specific isoform or disturbing the balanced expression of normally expressed isoforms in cancer cells (7). During tumor growth and development, and during oncogenesis, cells progress through various stages as they acquire additional oncogenic properties. These stages were described by Hanahan and Weinberg (8) in 2000 and in papers describing the features of cancer, which were updated in 2011 to include ten processes required for tumor development and progression to metastases. These processes were growth factor self-sufficiency; insensitivity to growth inhibitory signals; limitless replicative potential; the ability to evade apoptosis; the ability to sustain angiogenesis; the ability to invade tissues and metastasize; the ability to evade the immune system; the presence of inflammation; the tendency towards genomic instability; and deregulated metabolism. The results of recent studies indicate that alternative splicing

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Abbreviations: pre-mRNA, precursor messenger RNA; SSO, splice-switching oligonucleotides; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR

Key words: splice-switching oligonucleotides, gliomas, alternative splicing, apoptosis

regulates many of these processes involved in tumorigenesis and development (9-17). Accordingly, deregulated alternative splicing is considered to be a key feature of cancer and an opportunity to identify cancer biomarkers (18).

Because of its frequently occurring and important role in cancer, alternative splicing has emerged as an important target for molecular therapies. Therefore, many molecules have been designed and developed in order to inhibit cancer-specific isoforms or isoforms highly expressed in cancer cells, or switch the expression of specific isoforms as cancer cell-specific treatments (19,20). Splice-switching oligonucleotides (SSOs) are antisense oligonucleotides that modify alternative splicing by hybridizing to pre-mRNA sequences, which undergo splicing, and blocking access to the transcript by splicing factors, thereby redirecting the splicing machinery to the alternative pathway. The efficacy of SSOs has been established in various disease models, including β-thalassemia, Duchenne muscular dystrophy, spinal muscular atrophy, inflammatory diseases, and cancer (21,22).

In cancer, the first and most frequently cited demonstration of the antitumor effects of SSOs were of SSOs targeting Bcl-x pre-mRNA. Bcl-x is a member of the Bcl-2 family of proteins, which regulate the permeability of the outer membrane of mitochondria. Bcl-x undergoes splicing at two alternative 5' splice sites in exon 2, yielding two distinct proteins, Bcl-xL and Bcl-xS, with antagonistic properties (Fig. 1). Bcl-xL exerts anti-apoptotic effects by antagonizing and inhibiting the pro-apoptotic Bax and Bak proteins. Bcl-xL overexpression has been detected in several types of cancer. High Bcl-xL expression is correlated with decreased cellular sensitivity to chemotherapeutic reagents. Bcl-xS has pro-apoptotic effects by directly binding to and inhibiting the pro-apoptotic Bcl-xL and Bcl-2 proteins. High Bcl-xS expression was reported to induce apoptosis in cancer cells from patients with colon or stomach cancers (23,24).

Redirecting the pre-mRNA splicing of Bcl-x from Bcl-xL to Bcl-xS with an SSO had pro-apoptotic and chemosensitizing effects in various cancer cell lines. An SSO targeting Bcl-x pre-mRNA (termed Bcl-x SSO) that blocked the downsteam 5' alternative splice site in exon 2 of Bcl-x pre-mRNA redirected Bcl-x pre-mRNA splicing from Bcl-xL to Bcl-xS. Accordingly, it increased the expression of pro-apoptotic Bcl-xS and decreasing the expression of anti-apoptotic Bcl-xL in various cancer cell lines. Mercatante *et al* (25) reported that Bcl-x SSO, which targeted the downstream alternative 5'-splice site in exon 2 of Bcl-x pre-mRNA, shifted splicing from Bcl-xL to Bcl-xS in prostate and breast cancer cells *in vitro*. They also found that Bcl-xS protein, induced by the SSO, sensitized the cancer cells to treatment with ultraviolet- and γ-irradiation and chemotherapeutic drugs. In other studies, the same group showed that delivery of Bcl-x SSO using a lipid nanoparticle redirected Bcl-x splicing and reduced the tumor burden in melanoma lung metastases *in vivo* (26,27).

The effects of modifying a target gene's splicing using an SSO vary depending on the expression profile of the target cells. The differences in the cellular responses to Bcl-x SSO-induced modification of Bcl-x pre-mRNA splicing were mainly attributed to the endogenous Bcl-xL expression level. Tumor cells containing higher levels of Bcl-xL were more susceptible to the effects of Bcl-x SSO (9,25). To date, however, the effects of Bcl-x SSO on glioma have not been reported. Previous studies have reported that Bcl-xL is highly expressed in glioma, and confers resistance to chemotherapies (28). Therefore, we hypothesized that Bcl-x SSO can modulate alternative splicing of Bcl-x pre-mRNA and inhibit the growth of glioma cells.

In this study, we examined the effects of Bcl-x SSO on glioma cell lines. First, we measured the endogenous mRNA and protein expression of Bcl-xL in human glioma cell lines and a normal human astrocyte cell line. Then, we determined the effects of Bcl-x SSO on apoptosis and viability of these glioma cell lines. Finally, we measured the shift in expression from Bcl-xL to Bcl-xS in glioma cell lines treated with Bcl-x SSO.

Materials and methods

Ethics. The study was approved by the Ethics Committee of the China-Japan Union Hospital of Jilin University (Changchun, China).

Cell culture. Two human glioma cell lines (U87 and U251) and a normal human astrocyte cell line (HA1800) were purchased from Boster Biological Technology, Ltd. (Wuhan, China). The cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere at 37˚C. DMEM, FBS, and other tissue culture reagents were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China).

SSO preparation and transfection. 2'-O-methoxyethyl-phosphorothioate SSOs were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The Bcl-x SSO (5'-TGGTTCTTACCCAG CCGCCG-3') targeted the downstream 5' alternative splice site of exon 2 of Bcl-x pre-mRNA. An oligonucleotide (5'-GCTAT TACCTTAACCCAG-3') targeting human β-globin pre-mRNA was used as negative control SSO.

The U87 and U251 cells were plated in 6-well culture plates containing antibiotic-free DMEM at a density of $5.0x10^5$ cells/ml. At >60% confluence, the cells were transfected with SSO using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a HiFi-MMLV cDNA kit (Beijing Kang Century Biotechnology Co., Ltd., Beijing, China) and random hexamer primers. The primers were designed using Primer 5 software (Premier Biosoft, Palo Alto, CA, USA) and synthesized by GenScript Co., Ltd. (Nanjing, China). The primers 5'-AGCGTAGACAAGGAGATGCAGG-3' (forward) and 5'-GTGGATGGTCAGTGTCTGGTCA-3' (reverse) were used to amplify Bcl-xL, and the primers 5'-AGTAAAGCAAGCGC TGAGGGAG-3' (forward) and 5'-ACTGAAGAGTGAGCCCA GCAGA-3' (reverse) were used to amplify both Bcl-xL and Bcl-xS. PCR was performed using GoldStar Best DNA

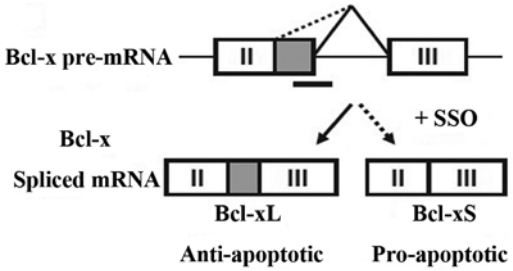

Figure 1. Schematic diagram of Bcl-x pre-mRNA alternative splicing by SSOs. Targeting the downstream or upstream alternative 5'-splice site in exon 2 of Bcl-x pre-mRNA yields anti-apoptotic Bcl-xL or pro-apoptotic Bcl-xS, respectively. The SSO targeting the downstream splice site redirects the splicing machinery to the upstream alternative splice site, and simultaneously decreases Bcl-xL expression and increases Bcl-xS expression.

Polymerase (Beijing Kang Century Biotechnology Co., Ltd.). The reaction conditions were as follows: 95˚C for 10 min followed by 40 cycles of degradation at 94˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 60 sec, and a final step of 72˚C for 10 min.

The PCR products were separated on a 10% non-denaturing polyacrylamide gel (Invitrogen Life Technologies) and bands were visualized on a Typhoon 9400 imager (GE Healthcare, Piscataway, NJ, USA). Images were quantified using ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA, USA). The relative abundance of Bcl-xS in each lane was determined by dividing the intensity of the 250-bp band (Bcl-xS) by the total intensities of the 452-bp (Bcl-xL) and 250-bp (Bcl-xS) bands.

Western blot analysis. Cells in all experimental groups were collected using a cell scraper. Total protein was extracted from cells using protein extraction reagent (Boster Bioengineering, Wuhan, China) containing 1 mM phenylmethanesulfonyl fluoride (PMSF; Roche Molecular Biochemicals, Indianapolis, IN, USA). The protein concentration was determined using the BCA protein assay (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Total protein was electrophoresed on a 15% sodium dodecyl sulfate-polyacrylamide gel and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Pall Gelman Laboratory Corporation, Ann Arbor, MI, USA). The membranes were blocked overnight using 5% skimmed milk powder at 4˚C. The membranes were washed in Tris-buffered saline containing Tween-20 (TBST) and incubated for 1 h at room temperature with primary antibodies against target proteins, followed by an additional TBST wash. The membranes were then incubated with appropriate secondary antibodies for 1 h at room temperature and washed again with TBST. The protein bands were detected by enhanced chemiluminescence.

Cell viability assay. The effects of Bcl-x SSO on the viability of human glioma cells were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells ($2x10^4$ cells/well) were cultured in 96-well culture plates for 1 day before SSO transfection. One day later, after the cells had adhered to the culture plates, they were transfected with Bcl-x SSO and control SSO. MTT solution (5 mg/ml, 20 μl; Sigma Chemical Co., St. Louis, MO, USA) was added to each well and the cells were cultured in a CO_2 incubator for 4 h. Next, the culture solution was removed and 150 μl of dimethyl sulfoxide was added to each well, and the plates were agitated at room temperature for 10 min. The optical density of each well was measured at 490 nm using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each experimental group was prepared in six duplicate wells. The mean values were calculated and growth curves were constructed.

Flow cytometry analysis. Apoptosis was evaluated by flow cytometry analysis (FCM). Cells from all experimental groups were digested in 0.25% trypsin and re-suspended in phosphate-buffered saline to prepare single-cell suspensions. The cell density was adjusted to $1x10^6$ cells/ml. Next, 5 μl of Annexin V-FITC and 5 μl of propidium iodide were added, and the cells were incubated for 30 min at 4˚C before flow cytometry.

Statistical analysis. Data are expressed as the mean ± standard deviation of experiments performed in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA) for multiple comparisons and unpaired t-tests for comparisons between pairs of groups. P-values of <0.05 were considered statistically significant.

Results

High expression of Bcl-xL in human glioma cell lines. We first determined the expression profile of Bcl-xL mRNA in two glioma cell lines (U87 and U251) and in a normal astrocyte cell line (HA1800) by RT-PCR. Bcl-xL mRNA expression was significant greater in U87 and U251 cells than in HA1800 (Fig. 2A and B). Likewise, western blot analysis revealed that Bcl-xL protein expression was also increased in U87 and U251 cells (Fig. 2C and D). The results indicate that Bcl-xL mRNA and protein levels are much greater in human glioma cell lines than in the control astrocyte cell line.

Bcl-x SSO inhibited the proliferation of human glioma cell lines. To shift the alternative splicing pathway of Bcl-x pre-mRNA from Bcl-xL to Bcl-xS, the U87 and U251 cells were transfected with Bcl-x SSO, which targeted the downstream 5' alternative splice site of exon 2, using the cationic lipid Lipofectamine 2000. To investigate whether Bcl-x SSO-mediated splice switching affected cell viability, MTT assays were performed 48 h after transfecting cells with different concentrations of SSO. The results showed that transfection with Bcl-x SSO significantly decreased the viability of U87 and U251 cells compared with untreated cells and cells transfected with a control SSO (Fig. 3). These results indicate that Bcl-x SSO inhibited the proliferation of human glioma cells.

Bcl-x SSO has pro-apoptotic effects on human glioma cell lines. To investigate whether Bcl-x SSO-mediated splice switching has pro-apoptotic effects on human glioma cell lines, flow cytometry was used to determine the rate of apoptosis. Transfection with Bcl-x SSO (200 nM) for 48 h significantly increased the apoptotic rate of U87 and U251 cells. By contrast, the control SSO did not affect the apoptotic

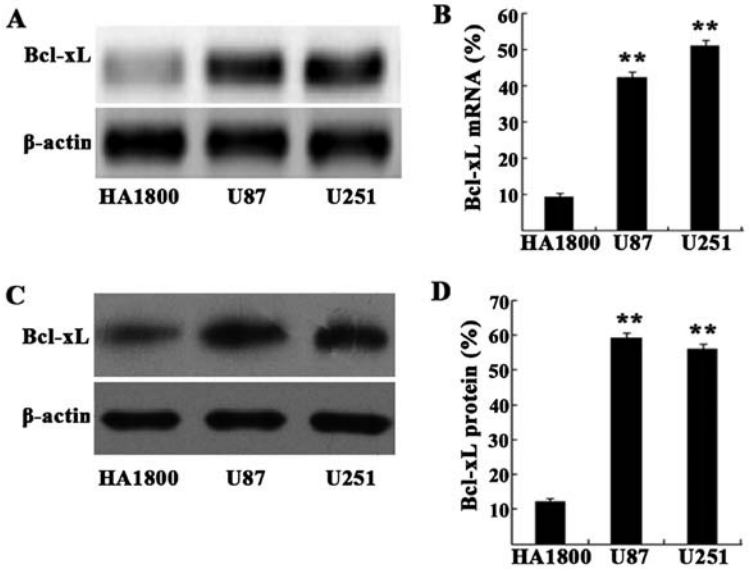

Figure 2. Comparison of Bcl-xL mRNA and protein expression levels in two human glioma cell lines (U87 and U251) and a normal human astrocyte cell line (HA1800). (A) RT-PCR analysis of Bcl-xL mRNA expression. (B) Relative Bcl-xL mRNA expression level normalized for β-actin mRNA. The mRNA expression of Bcl-xL was significantly greater in U87 and U251 cells than in HA1800 cells. (C) Western blot analysis of Bcl-xL protein expression. (D) Relative Bcl-xL protein expression level, normalized for β-actin protein. The protein expression of Bcl-xL was significantly greater in U87 and U251 cells than in HA1800 cells. Each independent experiment was repeated three times. $^{**}P<0.01$ vs. HA1800 cells. Error bars indicate the standard deviation of triplicate experiments.

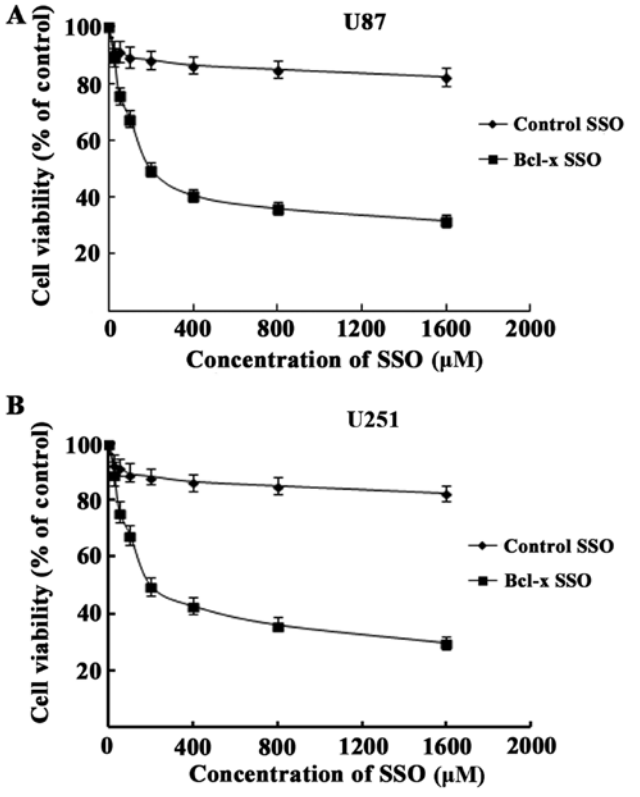

Figure 3. Bcl-x SSO inhibited the growth of human glioma cell lines. The inhibitory effects of Bcl-x SSO on the growth of U87 (A) and U251 (B) cells was determined by MTT assays. Cells were treated with Bcl-x SSO at different concentrations for 48 h. Each independent experiment was repeated three times.

rate of either cell line (Fig. 4). These results indicate that Bcl-x SSO has pro-apoptotic effects on human glioma cells.

Bcl-x SSO shifts Bcl-x splicing from Bcl-xL to Bcl-xS in human glioma cell lines. The shift in Bcl-x splicing in human glioma cell lines transfected with Bcl-x SSO (200 nM) for 48 h was determined by RT-PCR using primers to amplify both Bcl-xL and Bcl-xS. RT-PCR analysis of total RNA from U87 and U251 cells at 48 h after transfection showed that Bcl-x SSO caused a significant shift in splicing from the Bcl-xL to Bcl-xS pathway, as indicated by a shift in the ratio of these mRNAs. By contrast, the control SSO did not affect Bcl-x pre-mRNA splicing in either cell line (Fig. 5). Because a shift in the splicing pattern of Bcl-x pre-mRNA from Bcl-xL to Bcl-xS should lead to a change in Bcl-xL and Bcl-xS protein expression, we also conducted western blotting to determine the Bcl-xL and Bcl-xS protein expression levels. Consistent with the results of RT-PCR, the western blot analysis using anti-Bcl-xL and anti-Bcl-xS antibodies revealed a significant reduction in Bcl-xL protein expression and an increase in Bcl-xS protein expression (Fig. 6). Thus, the SSO-induced shift in Bcl-x splicing was confirmed in terms of the mRNA and protein expression levels of Bcl-xL and Bcl-xS.

Discussion

Gliomas are the most aggressive and lethal tumors of the central nervous system. In the past decades, the targeted therapy of glioma has not gained significant breakthrough, mainly due to the lack of an idea molecular target underlying gliomas (29). Alternative splicing is a key process involved in proteomic diversity and is essential for normal cell growth and development. However, deregulation of alternative splicing may occur and is implicated in various human diseases, including cancer. Aberrant alternative splicing is now considered an important event in cancer (1-6). Recent studies show that alternative

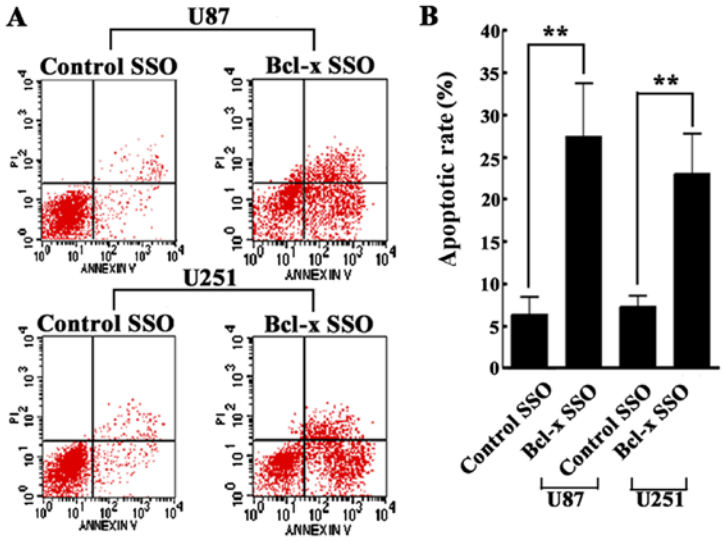

Figure 4. Pro-apoptotic effects of the Bcl-x SSO on human glioma cell lines. (A) Flow cytometry was performed to determine the apoptotic rate. (B) Quantification of the apoptosis rate (%) in U87 and U251 cells. **P<0.01 vs control cells. Error bars indicate the standard deviation of triplicate experiments.

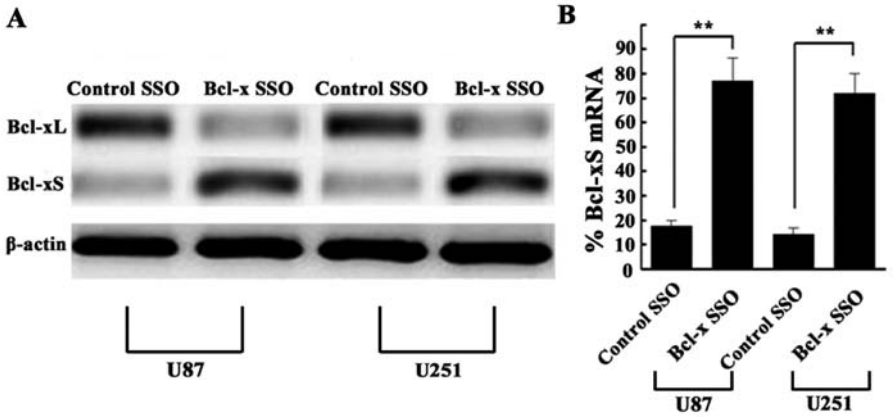

Figure 5. Transfection with the Bcl-x SSO shifted the splicing of Bcl-x mRNA from Bcl-xL to Bcl-xS in human glioma cell lines. (A) RT-PCR analysis of Bcl-xL and Bcl-xS mRNA expression levels. (B) Quantification of Bcl-xL and Bcl-xS mRNA expression levels. **P<0.01 vs. control cells. Error bars indicate the standard deviation of triplicate experiments.

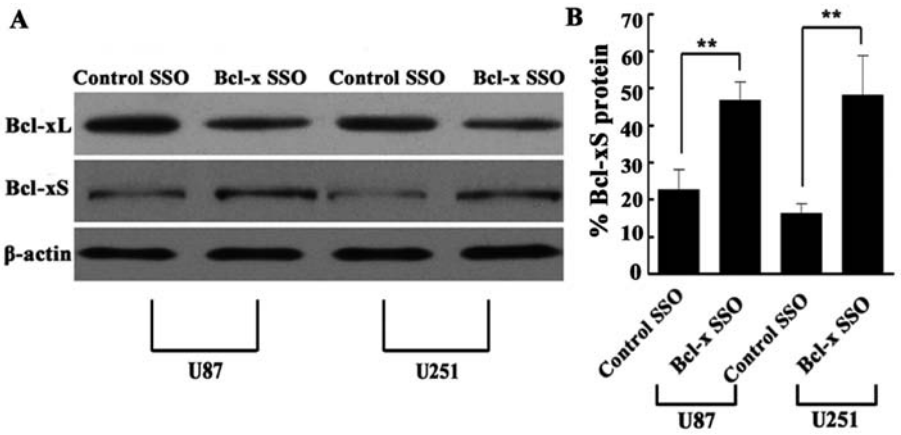

Figure 6. Transfection with the Bcl-x SSO shifted the splicing of Bcl-x protein from Bcl-xL to Bcl-xS in human glioma cell lines. (A) Western blot analysis of Bcl-xL and Bcl-xS protein expression levels. (B) Quantification of Bcl-xL and Bcl-xS protein expression levels. **P<0.01 vs control cells. Error bars indicate the standard deviation of triplicate experiments.

splicing also makes important contributions to the genesis and development of gliomas (30,31).

Thus, aberrant alternative splicing has emerged as an important target for molecular therapies, and methods to

manipulate alternative splicing are therapeutically valuable. Accordingly, SSOs have been developed to regulate alternative splicing by directing splice site selection. SSO are short oligonucleotides designed to anneal to a specific target region on pre-mRNA to interfere with pre-mRNA splicing. SSOs targeting an exon-intron junction may sterically block access to the splicing machinery, redirecting splicing reaction to an adjacent splicing site. Numerous studies have demonstrated the potential anticancer effects of SSO both *in vitro* and *in vivo* (20,32).

In cancer, the first and frequently quoted demonstration of antitumor efficacy of SSO was Bcl-x SSO targeting to Bcl-x pre-mRNA. Bcl-x SSOs block the downstream 5' alternative splice site in exon 2 of Bcl-x pre-mRNA and thereby redirect Bcl-x pre-mRNA splicing from Bcl-xL to Bcl-xS. Redirection of Bcl-x pre-mRNA splicing from Bcl-xL to Bcl-xS by SSOs had pro-apoptotic and chemosensitizing effects in various cancer cell lines. Mercatante *et al* were among the first to demonstrate the antitumor effects of SSOs (9). They reported that Bcl-x SSO initiated pro-apoptotic events and promoted cell death by decreasing Bcl-xL expression and increasing Bcl-xS expression in prostate cancer cells and breast cancer cells *in vitro*. They also reported that Bcl-xS, which was upregulated by SSO, sensitized the cancer cells to irradiation and chemotherapeutic drugs. Soon after, Bauman *et al* (26,27) demonstrated the *in vivo* antitumor efficacy of Bcl-x SSO. Using lipid nanoparticles, they administered Bcl-x SSO to a mouse model of metastatic melanoma. Bcl-x SSO efficiently redirected Bcl-x pre-mRNA splicing from Bcl-xL to Bcl-xS, and significantly reduced the tumor burden in mice with rapidly growing and highly tumorigenic lung metastases. However, no studies have investigated the antitumor effects of Bcl-x SSO on glioma cells, until now.

In another study by Mercatante *et al* (25), they found that the efficiency of splicing modulation and the corresponding antitumor effects of Bcl-x SSO were highly dependent on the expression profile of Bcl-xL. Tumor cells containing higher levels of Bcl-xL were more susceptible to the effects of Bcl-x SSO, which suggests that cancers with high Bcl-xL expression may show the greatest responses to Bcl-x SSO. Previous studies have revealed that Bcl-xL expression was elevated and contributed to chemotherapeutic resistance in glioma (28). In this study, in order to explore whether human gliomas are potential candidates for Bcl-x SSO therapy, we analyzed the mRNA and protein expression levels of Bcl-xL in two human glioma cell lines (U87 and U251) and a normal astrocyte cell line (HA1800). We found that Bcl-xL mRNA and protein expression levels were elevated in both human glioma cell lines, and were significantly higher in these cell lines than in HA1800 cells. Based on the results of previous studies and our results, we speculate that human glioma cell lines are good candidates for Bcl-x SSO therapy, which may modulate alternative splicing of Bcl-x pre-mRNA and inhibit glioma cell growth.

In our study, we designed the Bcl-x SSO to bind to the 5'-splice site of exon 2 in Bcl-x pre-mRNA. We also used a oligonucleotide targeting an aberrantly spliced human β-globin intron as a negative control SSO. The Bcl-x SSO and control SSO were modified using 2'-O-methoxyethyl-phosphorothioate and were delivered into U87 and U251 cells using a cationic lipid. We next examined the cellular effects of Bcl-x SSO on the human glioma cells. In these experiments, administration of Bcl-x SSO significantly reduced the replication rate of human glioma cells, demonstrating that Bcl-x SSO can inhibit the proliferation of glioma cells. We also examined the effects of Bcl-x SSO on cell apoptosis by flow cytometry, and the results revealed that Bcl-x SSO enhanced glioma cell apoptosis, whereas the control SSO had no effects on apoptosis. Finally, we determined the effects of Bcl-x SSO on switching the splicing from Bcl-xL to Bcl-xS in terms of the RNA and protein expression levels of both isoforms. RT-PCR and western blotting revealed that administration of Bcl-x SSO significantly reduced the mRNA and protein expression levels of Bcl-xL, and correspondingly increased the mRNA and protein expression levels of Bcl-xS. In other words, by targeting the alternative splicing of Bcl-x pre-mRNA, Bcl-x SSO reduced Bcl-xL expression and increased Bcl-xS expression at the mRNA and protein levels, and thereby promoted cancer cell apoptosis. Our data are consistent with the predicted mechanism of action of SSOs.

Unlike siRNA and conventional antisense oligonucleotides (ASOs), which degrade RNA via RNA-induced silencing complex (RISC) and RNase H-mediated cleavage, respectively, SSOs block sequences in pre-mRNA without causing RNA degradation. To achieve this, the SSO forms a very stable duplex with its pre-mRNA target sequence. This process can be encouraged by chemically modifying the oligonucleotide sugar-phosphate backbone to improve binding affinity and avoid RNase H cleavage, for example. The oligonucleotide sugar-phosphate backbone can be modified using 2'-O-methyl, 2'-O-methoxyethyl, phosphorodiamidate morpholino oligomers, and peptide nucleic acids (33,34). In our study, we modified the Bcl-xL SSO with 2'-O-methoxyethyl-phosphorothioate.

Two features of splicing modulation that distinguish this approach from downregulation of anti-apoptotic genes using ASO or siRNA are worth mentioning. First, Bcl-x SSO, which shifts the splicing of Bcl-x pre-mRNA from Bcl-xL to Bcl-xS, should be superior to Bcl-xL ASO and siRNA, which downregulate Bcl-xL mRNA expression. This is because Bcl-x SSO decreases Bcl-xL expression and concomitantly increases the expression of the antagonistic Bcl-xS, and thus amplifies the biological effects of the treatment. Second, Bcl-x SSO shows good specificity because its splicing modulation efficiency and antitumor effect are dependent on the expression of Bcl-xL. These properties suggest that cells showing higher Bcl-x expression are more susceptible to the effects of SSO, by greater upregulation of anti-apoptotic Bcl-xS expression. Accordingly, cells from aggressive cancers with higher Bcl-xL expression levels are likely to be more susceptible to SSO-induced apoptosis than healthy untransformed cells. This counterintuitive mechanism should also help to reduce the undesirable side effects associated with established chemotherapeutic drugs. Accordingly, cancers displaying high Bcl-xL expression and those that depend on Bcl-xL expression for survival represent good candidates for treatment with an SSO targeting Bcl-x. By contrast, ASOs and siRNA are less effective in cells showing high expression of the target gene owing to incomplete inhibition of the target gene in such cells (35,36). Here, we showed that Bcl-xL is highly expressed in human glioma cells, and we proposed the hypothesis that human glioma cells are susceptible to Bcl-xL SSO. We next confirmed that Bcl-x SSO modulates Bcl-x pre-mRNA splicing and has marked

pro-apoptotic effects in human glioma cell lines. We also found that Bcl-xL expression was significantly greater in the glioma cell lines than in the normal human astrocyte cell line. Accordingly, we may be able to find a suitable dosage of Bcl-x SSO that has marked antitumor effects on glioma cells without causing undesirable side effects on normal cells.

In conclusion, the present study was the first to explore the pro-apoptotic effects of Bcl-x SSO on glioma cell lines. The results showed that Bcl-x SSO modulated the alternative splicing of Bcl-x pre-mRNA from Bcl-xL to Bcl-xS in glioma cell lines. Bcl-x SSO had antitumor effects by inducing apoptosis and cell death in human glioma cell lines *in vitro*. These observations indicate that Bcl-x SSO may represent an efficient gene therapy for gliomas.

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