Potent Antitumor Activity in Experimental Hepatocellular Carcinoma by Adenovirus-Mediated Coexpression of TRAIL and shRNA against COX-2

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Abstract

Purpose: Recent studies have indicated that short hairpin RNA (shRNA) driven by RNA polymerase (Pol) II promoters can be transcribed into precursor mRNAs together with transgenes. It remains unclear, however, whether coexpression of shRNA and transgene from a single promoter is feasible for cancer therapy.

Experimental Design: In this study, we generated novel adenoviral vectors that permitted coexpression of shRNA against cyclooxygenase-2 (COX-2) and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) therapeutic gene from a cytomegalovirus promoter to evaluate whether silencing of COX-2 could increase the sensitivity of hepatocellular carcinoma to TRAIL.

Results: Our data showed that adenovirus vector Ad-TM, in which the shRNA was inserted into the 3′ untranslated region of the TRAIL gene, not only significantly suppressed COX-2 expression, but also expressed a high level of TRAIL. Moreover, infection with Ad-TM resulted in significant cytotoxicity in hepatocellular carcinoma cell lines. In contrast, it had no effect on normal liver cell line. Impressively, treatment of the established hepatocellular carcinoma tumors with Ad-TM resulted in complete tumor regression. This potent antitumor activity induced by Ad-TM was due to strong inhibition of COX-2 and high expression of TRAIL. Furthermore, using the shRNA and transgene coexpression adenovirus system, we showed that silencing of COX-2 increased the sensitivity of hepatocellular carcinoma to TRAIL through inhibition of Bcl-2 and Bcl-w.

Conclusion: This study indicated that adenovirus carrying shRNA and transgene expressed from a single promoter represented a potent approach for cancer therapy. Clin Cancer Res; 16(14); 3696–705. ©2010 AACR.

RNA interference (RNAi) is a fundamental cellular mechanism for silencing gene expression (1). For cancer therapy, RNAi has been used to silence the expression of oncogenes and growth factors or their receptors, which could result in inhibition of cell cycle and tumor angiogenesis and induction of cell apoptosis (2, 3). This technology has been regarded as an effective and useful approach for therapeutic applications of cancer (2, 4). Further reports indicated that the antitumor activity of RNAi could be enhanced if it was combined with therapeutic genes in different tumor models (5, 6). In this case, two independent vectors expressing short hairpin RNA (shRNA) and transgene respectively should be applied. Although this combined therapy showed great promise, it would induce cellular toxicity due to the application of two vectors if it is applied to patients. Recent studies have shown that shRNA could be coexpressed with transgene by incorporation of shRNA into untranslated regions (UTR) of transgene driven by RNA polymerase (Pol) II promoter. This expression system mimics endogenous microRNA expression in mammalian cells and displays efficient knockdown of gene expression (7–10). Therefore, simultaneous expression of shRNA and therapeutic gene by a single vector would be more flexible for cancer gene therapy.

It has been shown that tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising agent for therapy of cancer (4, 11). However, many malignancies including hepatocellular carcinoma have displayed a striking resistance to TRAIL therapy (12–14). The mechanisms of the resistance were related to TRAIL-induced NF-κB activation and upregulation of antiapoptotic molecules, Bcl-2 family, and inhibitor of apoptosis proteins (IAP) in hepatocellular carcinoma cells (11, 14–16). To restore hepatocellular carcinoma cell sensitivity to TRAIL, the combination of targeting NF-κB, XIAP, or antiapoptotic proteins with TRAIL achieved therapeutic benefit on hepatocellular carcinoma (4, 5, 14–18).
Antitumor Activity by TRAIL and COX-2 shRNA

Translational Relevance

Hepatocarcinogenesis is related to multiple dysregulation of gene expression. For therapeutic purposes, it is ideal to manage multiple targets involved in the pathogenesis of hepatocellular carcinoma. Recent studies have shown that coexpression of short hairpin RNA (shRNA) and therapeutic genes could be achieved by using a single promoter. In this study, we explored whether coexpression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and shRNA against cyclooxygenase-2 (COX-2) by adenoviral vector would be a useful approach for targeting apoptotic pathways in hepatocellular carcinoma cells. Our data indicated that this vector induced a high level of TRAIL expression and complete inhibition of COX-2 expression. It exhibited strong cytotoxicity to hepatocellular carcinoma cells in both in vitro and in vivo conditions. The possible mechanisms of antitumor activity have been illustrated. This study indicated that adenovirus-carrying shRNA and transgene expressed from a single promoter represented a potent approach for cancer therapy.

Cyclooxygenase-2 (COX-2) is an inducible gene, whose expression is undetectable in most normal tissues. In contrast to fetal hepatocytes, which expressed COX-2 in response to proinflammatory stimuli, adult hepatocytes failed to express COX-2 regardless of the type of challenge (19). However, overexpression of COX-2 was commonly related to different types of carcinomas, including hepatocellular carcinoma (20–23). It was evident that COX-2 could enhance hepatocellular carcinoma invasiveness by promoting hepatocellular carcinoma proliferation and tumor angiogenesis (24, 25). Therefore, inhibition of COX-2 could exhibit therapeutic potential to hepatocellular carcinoma. It has been shown that COX-2–selective nonsteroidal anti-inflammatory drugs (NSAID) could induce apoptosis and inhibition of angiogenesis in hepatocellular carcinoma (17, 22, 26, 27). COX-2 inhibition triggered expression of CD95, tumor necrosis factor (TNF)-R, and TRAIL-R1 and TRAIL-R2 death receptors. Although accumulating evidence suggests that NSAIDs sensitize cancer cells to TRAIL-induced apoptosis, it remains unclear whether it is COX-2 dependent or independent (17, 26, 28). Furthermore, NSAIDs could produce liver injury, which would limit their clinical applications (29, 30).

Silencing of COX-2 by RNAi would avoid the side effects induced by NSAIDs. Recent studies showed that downregulation of COX-2 expression via RNAi inhibited tumor cell proliferation and colony formation in vitro in different types of cancer cells (2, 23, 24). COX-2 silencing abolished the metastatic potential of the highly malignant breast cancer cells (21). Therefore, in this study we constructed an adenoviral vector with newly developed coexpression system to express shRNA against COX-2 and the therapeutic gene TRAIL and explored its antitumor potential in experimental hepatocellular carcinoma.

Materials and Methods

Cell cultures

BEL7404, Huh7, HepG2, and Hep3B (human hepatocellular carcinoma cell lines) were purchased from the Shanghai Cell Collection. AKIN-1 (human normal liver cell line) was provided by Dr. Strom SC (University of Ulm; ref. 31). HEK293 (human embryonic kidney cells) was obtained from Microbix Biosystems, Inc. BEL7404, Huh7, HepG2, Hep3B, and HEK293 were cultured in DMEM (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL) at 37°C under a 5% CO2 condition.

Generation of plasmids and recombinant adenovirus

The miR30-based COX-2 targeting sequence (5′-CAGATCCAGAGGCTCATAA-3′) was obtained from RNAi Codex (http://codex.cshl.org), and a miR30-based nonsilencing control shRNA specific for dsRed protein (5′-GTGATGTC-CAGCCTTGGAGG-3′) was used (32). The sequences of COX-2-shRNA, dsRed-shRNA, and the intron imitated by Du et al. (8) were synthesized. To generate pShuttle-CMV-shRNA-COX-2, pShuttle-CMV-Control, and pShuttle-CMV-Intron, the synthesized COX-2-shRNA, dsRed-shRNA, and the intron DNA were annealed and cloned into pShuttle-CMV by SalI/NotI, BglII/HindIII, and KpmI/XhoI, respectively. To generate pShuttle-CMV-Intron-shRNA-COX-2, the synthesized COX-2-shRNA were also annealed and cloned into pShuttle-CMV-Intron by SalI/NotI. Two TRAIL fragments, TRAIL fragment-1 and TRAIL fragment-2, were separately amplified with two pairs of primers: pair 1 (forward: 5′-CGGTTACGATATCTGAGCAACTAA-AAGGCCCC-3′; reverse: 5′-TTGTACCCGGCCGAGCATGGAAGGTG-3′) and pair 2 (forward: 5′-GGCTCAGGGGGCCACCATGGCTATGAGGAGG-3′; reverse: 5′-GGAAGCTTGTAGACCAAAAGGGCCCGGA-3′). Both TRAIL fragments were cloned into pMD18-T Vectors. TRAIL fragment-1 was excised by KpnI and cloned into pShuttle-CMV-Intron-shRNA-COX-2 and pShuttle-CMV to obtain pShuttle-CMV-TRAIL-Intron-shRNA-COX-2 and pShuttle-TRAIL. TRAIL fragment-2 was excised by XhoI/HindIII and cloned into pShuttle-CMV-Intron-miRNA-COX-2 to generate pShuttle-CMV-Intron-miRNA-COX-2-TRAIL. All plasmid constructs were confirmed by digestion with restrictive enzymes, PCR, and DNA sequencing.

Recombinant adenoviral vectors Ad-Control, Ad-shRNA-COX-2, Ad-TRAIL, Ad-TM, Ad-TIM, and Ad-IMT were generated and purified according the standard protocol. The titration of recombinant adenovirus was done with TCID50 assay on HEK293 cells.
Western blotting

Total proteins were separated on 12% polyacrylamide gels and transferred onto 0.45 μm nitrocellulose and blocked with 5% bovine serum albumin. The membranes were incubated with primary antibodies anti-COX-2, anti-TRAIL, anti-caspase-3, anti-caspase-8, anti-poly(ADP-ribose) polymerase (PARP), anti-Bcl-2, anti-Bcl-W, and anti-β-actin (Santa Cruz), and followed by the addition of antirabbit IR dye 700, antigoat IR dye 700, and antimouse IR dye 800 (Li-Cor). The fluorescent signal was revealed through the Odyssey IR imaging system (Li-Cor).

Cytotoxicity assay

Cells were plated in 96-well plates and treated with various adenoviruses. At the indicated times, medium was removed and fresh medium containing 0.5 mg/mL MTT was added to each well. Cells were incubated at 37°C for 4 hours and absorbance was measured on TECAN DNA expert (TECAN) at 595 nm.

Tumor xenograft in nude mice

All animals used in this study were maintained at institutional facilities and received human care according to the criteria outlined in the Guide for the Care and
Use of Laboratory Animals. Male BALB/c nude mice (4-5 weeks of age) were obtained from the Animal Research Committee of the Institute of Biochemistry and Cell Biology (Shanghai, China). Mice were inoculated s.c. with BEL7404 cells (2 x 10⁶). When the tumor volume reached 100 to 150 mm³, the animals were randomly divided into four groups. Intratumoral injection of different adenoviruses (1 x 10⁸ PFU/dose) in 50 μL of PBS was done once every other day for a total of five times. The tumor size was measured using caliper.

Fig. 2. Construction and characterization of the adenoviral vectors coexpressing both shRNA and transgene. A, constructs of adenoviral vectors. Ad-TM, miRNA-based shRNA-COX-2 was directly inserted in the 3'UTR of TRAIL gene; Ad-TIM, miRNA-based shRNA-COX-2 was inserted in the intron of β-globin embedded in the 3'UTR of the TRAIL gene; Ad-IMT, miRNA-based shRNA-COX-2 was inserted in intron of β-globin embedded in the 5'UTR of the TRAIL gene. B, expression of COX-2 and TRAIL in Huh7 after infection with adenoviruses. Huh7 cells were infected with Ad-TM, Ad-TIM, Ad-IMT, Ad-shRNA-COX-2, Ad-TRAIL, or Ad-Control at MOI of 50. After 48 hours the cells were collected for COX-2 and TRAIL determination by Western blotting. C, expression of COX-2 and TRAIL in both tumor and normal cell lines after infection with adenoviruses. The hepatocellular carcinoma tumor cells Huh7 and BEL7404 and liver normal cells AKN-1 were infected with Ad-TM, Ad-TRAIL, or Ad-Control at MOI of 50. After 48 hours the cells were collected for COX-2 and TRAIL determination by Western blotting.
The tumor volume (mm³) was calculated as follows: (length × width²)/2.

**Immunohistochemistry**

Deparaffinized tumor sections (on 6 days after treatment) were stained with H&E or incubated with anti-COX-2 and anti-TRAIL antibodies. The staining signals were amplified by a biotinylated peroxidase-conjugated streptavidin system (Bio-Genex Laboratories).

**Propidium iodide/Hoechst 33342 staining**

Cells (1 × 10⁵) were cultured in each well of 6-well plates. Ten hours later, the cells were infected with Ad-TM, Ad-TRAIL, or Ad-Control. Forty-eight hours after infection, the medium was replaced with PBS and then the cells were stained with Hoechst 33342 (25 μg/mL) and propidium iodide (PI; 1 μg/mL). The percentage of apoptotic cells was analyzed with fluorescenceactivated cell sorter (FACS). Hoechst 33342 and PI were...
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Expression of COX-2 in hepatocellular carcinoma cells

To determine the status of COX-2 expression in hepatocellular carcinoma, we analyzed expression of endogenous COX-2 in four hepatocellular carcinoma cell lines (Huh7, Hep3B, BEL7404, and HepG2) and a normal liver cell line (AKN-1) by Western blotting. As shown in Fig. 1A, hepatocellular carcinoma cell lines expressed high levels of COX-2. In contrast, COX-2 protein could not be detected in the normal liver cell line. These data suggested that overexpression of COX-2 may correlate with hepatocellular carcinoma resistance to TRAIL and promote tumor cell growth.

Activation of caspase-3 by inhibition of COX-2 in hepatocellular carcinoma cells

To silence COX-2 expression, we constructed a recombinant adenovirus carrying a miRNA-based shRNA against COX-2, Ad-shRNA-COX-2, to inhibit COX-2 expression in hepatocellular carcinoma cell lines (Fig. 1B). Our data showed that expression of COX-2 was dramatically reduced after infection of hepatocellular carcinoma cells with Ad-shRNA-COX-2 (Fig. 1C). The high level of activated caspase 3 and cleaved PARP protein were observed in all hepatocellular carcinoma cells (Huh7, Hep3B, HepG2, and BEL7404) after infection with Ad-shRNA-COX-2, suggesting that downregulating COX-2 could induce apoptosis in hepatocellular carcinoma cells (Fig. 1D).

Characterization of adenoviral vectors for coexpression of shRNA and transgene in vitro

Recent studies suggest that coexpression of miRNA-based shRNA and transgene in a monocistronic transcript could lead to effective processing of the shRNA and translation of protein from the mRNA (7, 9, 33). To achieve maximum efficiency of COX-2 silencing and TRAIL expression in hepatocellular carcinoma cells, three adenoviral vectors (Ad-TM, Ad-TIM, and Ad-IMT) were generated for coexpressing miRNA-based shRNA and transgene (Fig. 2A). To compare the efficiency of shRNA-mediated silencing of COX-2 and expression of TRAIL, we infected Huh7 cells with three adenoviral constructs, and the expression of COX-2 and TRAIL was measured. As shown in Fig. 2B, the level of COX-2 expression was comparable in the cells infected with Ad-control, Ad-TRAIL, and cells without infection. In contrast, infection with Ad-TM and Ad-IMT resulted in slight reduction in COX-2 gene expression. The expression of COX-2 was almost undetectable in cells infected with Ad-TM and Ad-shRNA-COX-2 (Fig. 2B). The high and comparable level of TRAIL expression was only observed in the cells infected with Ad-TM and Ad-TRAIL. Low level of TRAIL expression was observed in the cells infected with Ad-TIM and Ad-ITM (Fig. 2B). This result indicated that Ad-TM could induce efficient silencing of COX-2 and expression of TRAIL. To further characterize Ad-TM, we infected Huh7 and BEL7404 hepatocellular carcinoma cells and AKN-1 normal liver cells with Ad-TM, Ad-TRAIL, or Ad-control. Our data showed that the infection with Ad-TM resulted in comparable expression of TRAIL as compared with cells infected with Ad-TRAIL, but it induced dramatic inhibition of COX-2 gene expression in hepatocellular carcinoma cells (Fig. 2C).

Inhibition of hepatocellular carcinoma cell growth by Ad-TM in vitro

To evaluate whether Ad-TM could impede hepatocellular carcinoma cell proliferation, we did cell viability assay in the hepatocellular carcinoma cells after infection with adenoviral vectors. As shown in Fig. 3, both Ad-TRAIL and Ad-shRNA-COX-2 could reduce cell viability in hepatocellular carcinoma cells. However, there was significant reduction in cell viability in tumor cells infected with Ad-TM, as compared with cells infected with Ad-TRAIL and Ad-shRNA-COX-2. These results suggested that COX-2 silencing sensitized hepatocellular carcinoma cells to TRAIL. In contrast, neither Ad-TM nor Ad-TRAIL nor Ad-shRNA-COX-2 exhibited cytotoxicity in normal liver cells. These data indicated that Ad-TM had enhanced...
antitumor activity in hepatocellular carcinoma cells and maintained low toxicity to normal liver cells.

**Antitumor efficacy in animal model with the established hepatocellular carcinoma tumors**

To know the antitumor potency of Ad-TM in vivo, we treated the established hepatocellular carcinoma tumors in an experimental animal model by intratumoral injection of Ad-TM, Ad-TRAIL, and Ad-control. Our data showed that animals receiving PBS experienced progressive tumor growth. Treatment with Ad-control resulted in minor inhibition of tumor growth (Fig. 4). In contrast, treatment with Ad-TM resulted in significant inhibition of tumor growth, compared with that induced by Ad-TRAIL and Ad-control ($P < 0.01$; Fig. 4). All animals treated with Ad-TM had complete tumor regression without obvious toxicity.

Immunohistochemistry analysis showed that expression of COX-2 was dramatically reduced in tumor sections from animals treated with Ad-TM, compared with those treated with PBS and Ad-control. In contrast, upregulation of TRAIL was observed only in tumor sections from animals treated with Ad-TM (Fig. 5). Pathologic analysis revealed that the necrotic area could be found in tumors treated with Ad-TM, whereas necrotic area was hardly observed in tumors treated with Ad-control (Fig. 5). These results indicated that treatment of adenoviral vectors carrying coexpression cassette of both transgene and shRNA resulted in high transgene expression and endogenous gene silencing in vivo.

**COX-2 silencing enhances TRAIL-mediated apoptosis via inhibition of Bcl-2 and Bcl-w**

Our results showed that the combination of COX-2 inhibition and TRAIL expression can induce significant cytotoxicity *in vitro* and *in vivo*. Our data further showed that there was increased apoptotic rate in hepatocellular carcinoma cells after infection with Ad-TM, as compared with Ad-TRAIL infection (Fig. 6A). These data were consistent with above observations and suggested that inhibition of COX-2 could enhance apoptosis induced by TRAIL.

To show the molecular mechanisms related to enhanced cell apoptosis to TRAIL by inhibition of COX-2, we examined the expression of Bcl-2 and Bcl-w, important inhibitors of apoptosis. Our data showed that both Bcl-2 and Bcl-w expression was high in all hepatocellular carcinoma cell lines (Fig. 6B). Infection of hepatocellular carcinoma cells *in vitro* with Ad-TM resulted in dramatic inhibition of Bcl-2 and Bcl-w expression, as
compared with cells infected with Ad-TRAIL or Ad-Control (Fig. 6C). The data from in vivo study also showed the decreased expression of Bcl-2 and Bcl-w in hepatocellular carcinoma tumors treated with Ad-TM (Fig. 6C). It implicated that Bcl-2 and Bcl-w were involved in apoptosis triggered by Ad-TM, and Ad-TM can induce apoptosis through both intrinsic and extrinsic pathways (Fig. 6D).

**Discussion**

Previous studies showed that shRNA and transgene carried by lentiviral vector can be transcribed into precursor mRNA where shRNA is embedded in the 3′UTR or in the intron of transgene (3, 8–10). As one of the best vehicles in the field of cancer gene therapy, adenovirus has been...
used to deliver shRNA and therapeutic genes. Previous studies from our laboratory and those of others showed that combined therapy with adenoviruses carrying shRNA and therapeutic genes could achieve better antitumor activity (5, 34). In this study, we generated adenoviral vectors that permitted coexpression of shRNA and transgene from a single Pol II promoter for cancer therapy. To get an efficient system, we initially constructed three adenoviral vectors, Ad-TM, Ad-TIM, and Ad-IMT. Both Ad-TIM and Ad-IMT did not induce COX-2 silencing significantly and they only expressed low level of TRAIL. It may be possible that the split of the intron was not complete. On the other hand, Ad-TM significantly silenced the endogenous COX-2 expression in tumor cells and expressed a high level of TRAIL. It may be possible that the split of the intron was not complete. On the other hand, Ad-TM significantly silenced the endogenous COX-2 expression in tumor cells and expressed a high level of TRAIL in both in vitro and in vivo conditions. This was probably because miR30, which was used as backbone of shRNA in this study, can be spliced efficiently (35). These results indicate that miRNA-based shRNA placed in 3′UTR of transgene is an efficient system for coexpression of shRNA and transgene simultaneously. Because efficient coexpression of shRNA and transgenes is driven by Pol II promoter, it is possible to apply tissue-specific and inducible coexpression of shRNA and transgene.

TRAIL is proposed as a promising antitumor agent that can induce apoptosis in tumor cells but not in normal cells (11, 36, 37). In addition, tumor metastases and invasion occur when the TRAIL-induced apoptosis pathway is impeded (11, 16, 37). Recent studies suggested that inhibition of COX-2 increased the sensitivity of hepatocellular carcinoma cells to TRAIL (17, 38, 39). In this study, our data showed that Ad-TM showed dramatic hepatocellular carcinoma cytotoxicity via COX-2 gene silencing coupled with TRAIL expression in both in vitro and in vivo conditions. On the other hand, Ad-TM did not elicit cytotoxicity to normal liver cells. Impressively, treatment of established hepatocellular carcinoma with Ad-TM resulted in complete tumor regression. This strong antitumor activity could be related to the high level of tumor cell death induced by the combined effect of COX-2 inhibition and TRAIL expression. These data indicate that Ad-TM is a promising agent for therapy of hepatocellular carcinoma.

Furthermore, our data showed that Ad-TM caused increased apoptotic ratio via inhibition of Bcl-2 family proteins. It was consistent with the previous observation that inhibition of COX-2 could enhance TRAIL-mediated apoptosis (17, 38, 39). Moreover, our data showed that Bcl-w is involved in the machinery, as is Bcl-2, which was not referred to in previous studies. Bcl-w is an important member in the Bcl-2 family and generally exhibited high expression in TRAIL-resistant tumor cells (40, 41). Recent reports showed that Bcl-w is a target protein of hepcatic-specific microRNA-122, which plays an important role in induction of apoptosis in hepatocellular carcinoma (42, 43). This indicates that Bcl-w is involved in intrinsic apoptotic pathway in hepatocellular carcinoma. Therefore, it could be hypothesized that adenovirus vector Ad-TM induces apoptosis through both intrinsic and extrinsic pathways. Ad-TM can be transcribed into a pri-miRNA-COX-2 and a TRAIL mRNA in the nucleus. Then the pri-miRNA-COX-2 can be processed into shRNA and directed to a RNA-induced silencing complex to cleave COX-2 mRNA. After COX-2 silencing, Bcl-2 and Bcl-w are downregulated so that the intrinsic apoptosis pathway is induced. On the other hand, TRAIL is also translated and then targets the TRAIL-R1 and R2 on the cell membrane. It activates the extrinsic apoptosis pathway (Fig. 6D). In addition to induction of apoptosis, other mechanisms may be involved in regression of tumors by combined therapy. It has been shown that high level of COX-2 expression contributed to tumor angiogenesis and inhibition of COX-2 activity resulted in decreased angiogenesis by suppression of vascular endothelial growth factor expression (44). Our previous study has shown that treatment of cancer by TRAIL and inhibitor of angiogenesis could induce tumor regression (45). Therefore, inhibition of tumor angiogenesis may participate in tumor eradication treated by inhibition of COX-2 expression and TRAIL. Further study is needed to clarify this mechanism.

Taken together, we generated an adenoviral vector to express both shRNA against COX-2 and therapeutic gene TRAIL from a single Pol II promoter. We showed that Ad-TM exhibited significant hepatocellular carcinoma cytotoxicity in both in vitro and in vivo conditions, indicating that Ad-TM is a potential agent for therapy of hepatocellular carcinoma. Moreover, our results exhibited that COX-2 silencing enhanced TRAIL-induced apoptosis involving both Bcl-2 and Bcl-w. This study provides promise for the development of a novel cancer gene therapeutic strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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