

Enhanced Therapeutic Efficacy by Simultaneously Targeting Two Genetic Defects in Tumors

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Targeting tumor-specific gene abnormalities has become an attractive approach in developing therapeutics to treat cancer. Overexpression of *Bcl2* and mutations of *p53* represent two of the most common molecular defects in tumors. In the nucleus, *p53* induces cell cycle arrest, while it interacts with *Bcl2* outside of the nucleus to regulate signal pathways involved in apoptosis. To potentiate antitumor activity, we tested a "double target" approach to antitumor therapy by combining H101, a recombinant oncolytic adenovirus that targets the inactive *p53* in tumors, with a small interfering RNA (siBCL2) that targets *Bcl2*. In cell culture, the combined treatment significantly enhanced apoptosis and cytotoxicity as compared with treatment with either H101 or siBCL2 alone. In animals carrying tumor xenographs, combined H101 and siBCL2 treatment significantly inhibited tumor growth and prolonged survival. At the end of the study, all animals in the combined therapy group survived and two of the five animals showed complete eradication of their tumors. Interestingly, siBCL2 treatment increased H101 viral replication in both treated cells and tumor tissues. Simultaneously targeting two tumor-specific gene abnormalities using an oncolytic adenovirus and siRNA potentiates total antitumor activity.

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INTRODUCTION

Conventional chemotherapeutic agents, though often effective, are highly toxic because of their lack of selectivity for cancer cells. As a result, efforts have focused on developing interventions that can target tumor-specific genes, using techniques that include tumor-selective replicating viruses and siRNA.

The *p53* tumor suppressor, the "guardian of the genome", is the most frequently mutated gene in cancer.¹ Loss of function of the wild-type *p53* allows cells to evade apoptosis, thereby promoting tumor progression. A clinically validated approach to therapy is to restore *p53* function by replacing the mutant gene using a replication-deficient virus that carries the wild-type *P53* (Ad5CMV-*p53*).

Restoration of *P53* function in tumor cells may induce apoptosis and sensitize cells to cytotoxic killing, thus improving the therapeutic response. Clinical studies have shown that intratumoral injection of Ad5CMV-*p53* resulted in wild-type *P53* expression in various tumor tissues, leading to selective death of cancer cells (see reviews ref. 2–4).

Another virus-based strategy takes advantage of the fact that the replication and production of adenoviral progeny require the cell cycle gatekeeper *p53* to be in an inactive status, which is commonly observed in tumors due to mutation or epigenetic silencing of the gene. Adenoviruses infect quiescent cells and induce them to enter the S phase of the cell cycle so that viral DNA replication can proceed. Adenovirus early genes encode proteins that have several functions aimed at activating the S phase of the cell cycle. One viral early gene, *E1B*, which encodes a 55-kDa protein (E1B 55K), is essential to virus replication. E1B interacts with cellular *p53* and inactivates it to allow viral replication. ONYX-015, a mutant adenovirus that lacks the *E1B 55K* gene, can only replicate and lyse tumor cells that have inactivated *P53*, sparing normal cells that retain wild-type *p53* function.⁵ Clinical trials in patients with recurrent head and neck cancer, metastatic colorectal cancer, or pancreatic cancer have shown that ONYX-015 is safe and has significant anti-tumor effect in at least a fraction of the patients, when it is used alone or combined with chemotherapy (review refs 2,6–8).

In China, an oncolytic adenovirus called H101 has been clinically approved for the treatment of several malignancies.⁹ In this virus, both *E1B* and *E3* are deleted, so that it selectively infects and kills tumor cells through viral oncolysis.¹⁰ Without E1B to inactivate *p53*, H101 adenovirus cannot replicate and lyse normal cells where *p53* is active. Thus, H101 does not have significant cytopathic effects on normal cells. However, H101 has limited efficacy as a monotherapy in clinical practice. Tumors show great variation in regard to their susceptibility to oncolytic adenoviruses, presumably because E1B has other functions in addition to targeting *p53*, such as viral RNA export and inhibition of host protein synthesis. Other studies have shown that the replication of this type of oncolytic adenovirus in human tumor cells is actually independent of *p53* status.^{11,12} Thus, in practice, *p53*-based therapeutic strategies are often combined with conventional cancer therapy to minimize development of therapy resistance.

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It is interesting to note that p53 not only functions in the nucleus to induce cell cycle arrest, but also has an “extranuclear” proapoptotic function as it partners with *Bcl2*,^{13,14} another well-established tumor target gene. By forming complexes with *Bcl2* on the outer mitochondrial membrane, p53 directly disrupts *Bcl2*/Bax to regulate apoptosis. *Bcl2* expression is negatively regulated by p53 and is upregulated in p53-deficient cells.¹⁵ On the other hand, *Bcl2* phosphorylation blocks this p53 interaction and preserves its antiapoptotic activity.¹⁶ Overexpression of antiapoptotic *Bcl2* or *Bcl-xL* abrogates stress signal-mediated mitochondrial p53 accumulation and apoptosis.¹⁴ Thus, p53 and *Bcl2* are tightly coordinated in balancing “Ying-Yang” pathways of apoptosis. *Bcl2* is overexpressed in a wide range of human tumors where it promotes tumor formation by impeding apoptosis.^{17,18} High levels of *Bcl2* expression in tumors is associated with faster time to relapse, shorter survival, and other indications of poor clinical outcome.¹⁹ Thus, the concurrence of p53 and *Bcl2* genetic defects in tumors may represent a molecular “double-hit” related to tumor growth.

We propose to enhance anticancer efficiency by testing a “double-targeted” therapeutic approach. Suppression of *Bcl2* expression by antisense and RNAi compounds inhibited tumor growth and enhanced apoptosis in a variety of animal models.^{20,21} We have previously used RNAi and methylated oligonucleotides^{22,23} to knock down *Bcl2*. Using plasmid vectors expressing short hairpin RNAs (shRNAs), we showed that silencing *Bcl2* improved the antitumor

effect of the chemotherapeutic agent 5-FU.²² In this communication, we have examined the effect of simultaneously targeting two tumor-related genetic defects: overexpressed *Bcl2* and mutant p53. We incubated the H101 oncolytic adenovirus in addition to *Bcl2* RNAi with a cervical cancer cell line HeLa-S3 that overexpresses *Bcl2* but is deficient in p53.²⁴ Preclinical studies demonstrated that HeLa-S3 was very sensitive to H101 oncolytic treatment. We found that this “double target” therapy significantly enhanced anticancer efficiency both *in vitro* and *in vivo*, presumably by enhanced viral replication and increased apoptosis in tumors.

RESULTS

Targeted silencing of *Bcl2* by synthetic siRNA

Previously, we silenced *Bcl2* transcription using a plasmid DNA vector that delivered two short *Bcl2* hairpin RNAs.²² On the basis of our previous data, we synthesized two siRNAs that target two different positions of *Bcl2* mRNA as shown in **Figure 1a**. The first *Bcl2* siRNA (siBCL2-X) that targets the 5' region of the *Bcl2* mRNA was completely ineffective in silencing *Bcl2* gene expression (data not shown). The second *Bcl2* siRNA (siBCL2), however, was found to effectively suppress *Bcl2* as determined by semiquantitative PCR (**Figure 1b**, lane 6) as compared with control siRNA (siNC, lane 5) and control cells (phosphate buffered saline (PBS), lane 4). *Bcl2* silencing by siBCL2 was also confirmed at the protein production level using Western blot analysis (**Figure 1c**, lane 6 versus lanes 5, 4).

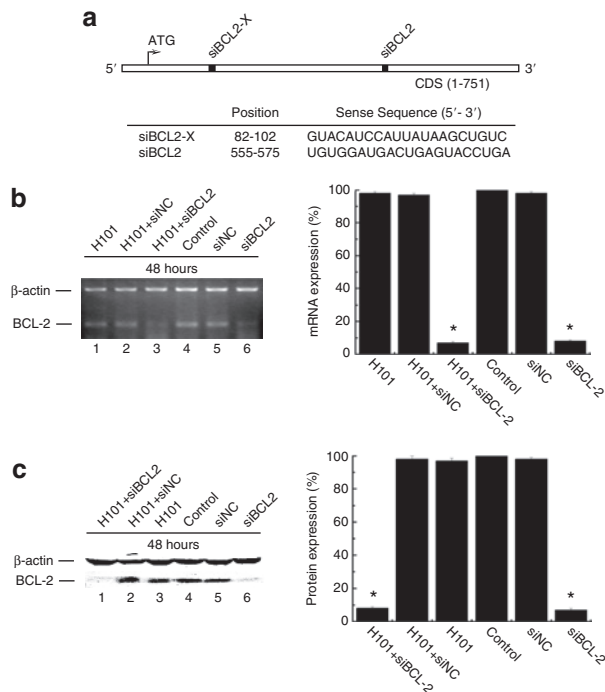


Figure 1 *Bcl2* gene knockdown by siRNA. **(a)** Schematic representation and location of two *Bcl2*-specific siRNAs used in this study. **(b)** Semi-quantitative RT-PCR analysis of *Bcl2* transcripts in HeLa-S3 cells. Left panel: PCR gel. Right panel: PCR bands were scanned and normalized over the internal control β -actin. The value of PBS control was set as 100%. **(c)** Western Blot analysis of *Bcl2* protein expression in HeLa-S3 cells. All experiments were performed 48 hours after siBCL2 (50 nmol/l) transfection with or without H101 infection (multiplicity of infection (MOI) = 100). β -actin was used as the internal control for normalization. The value of PBS control was set as 100%.

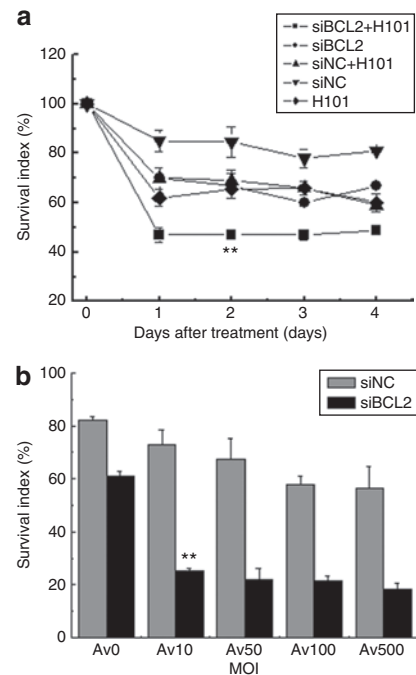


Figure 2 Growth inhibition of HeLa-S3 cells. **(a)** The combined treatment of siBCL2 and H101. Cell growth was measured by MTT assays at days 1, 2, 3, 4 after co-treatment of siBCL2 (50 nmol/l) and H101 (multiplicity of infection (MOI) = 100). **(b)** Treatment of siBCL2 with varying doses of H101 adenovirus. Cell growth was measured at day 2 after treatment with 50 nmol/l siBCL2 or siNC. H101 infection was performed at MOIs of 0, 10, 50, 100, and 500. All data are presented as means \pm SD. of three independent experiments. $P < 0.01$: as compared with untreated HeLa-S3 cells.

Oncolytic adenovirus H101 uses an entirely different mechanism to target tumor cells, and therefore we wanted to test whether viral infection would affect siRNA-mediated *Bcl2* silencing. Cells were co-treated with siBCL2 and H101. *Bcl2* gene expression was examined by both semiquantitative RT-PCR and Western blotting. As expected, viral infection alone or with the control siRNA (siNC) did not significantly inhibit *Bcl2* expression. *Bcl2* expression was markedly inhibited by siBCL2 or siBCL2 plus H101 (Figure 1b,c). This experiment demonstrates that adenoviral infection does not alter the gene silencing caused by synthetic siRNA in cancer cells.

Enhanced cytotoxicity of HeLa-S3 tumor cells by the combined treatment of H101 and *Bcl2* siRNA

H101 and siBCL2 inhibit tumor cell growth by targeting two different oncoprotein pathways. We were interested in examining whether these two treatments would significantly enhance anti-tumor effects in tumor cells. As compared with the control group, cells treated with the monotherapy of siBCL2 or H101 showed moderate reduction of cell survival (Figure 2a), starting from the second day following the treatment and maintained thereafter. The control siRNA (siNC) reduced cell growth by 20%. However, in the group treated with combined H101 and siBCL2, cell growth was significantly inhibited, indicating an anticancer augmentation of the combined tumor therapy.

We then examined the effect of using various titers of H101 in siBcl-transfected cells. In cells treated with the control siRNA (siNC), increasing H101 titers showed a slight dose-response inhibition of cell growth (Figure 2b). In cells treated with *Bcl2* siRNA (siBCL2) plus H101, however, there was a significant reduction in cell survival, even with a very low titer of the oncolytic adenovirus (multiplicity of infection = 10). Further increases in H101 titers did not dramatically increase cell inhibition. These data suggest that *Bcl2* siRNA treatment can reduce the required dose of oncolytic adenovirus H101.

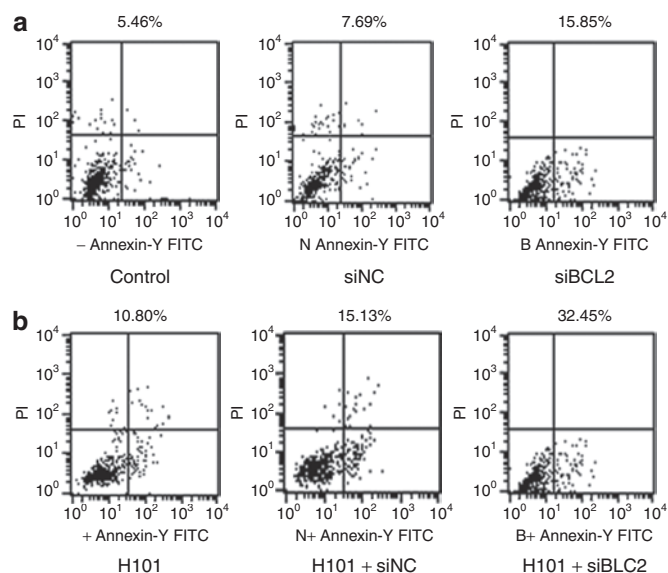


Figure 3 Apoptotic activity of in HeLa-S3 cells. Apoptosis were measured by flow cytometric analysis at 48 hours after co-treatment of siBCL2 and H101.

Enhanced apoptosis by combined siBCL2 and H101 treatment

To examine apoptosis, siBCL2 was transfected into HeLa-S3 cells using Lipofectamine 2000. Forty-eight hours after transfection, apoptosis were measured using an Annexin V-FITC apoptosis kit and flow cytometric analysis. As seen in Figure 3a, siBCL2 induced apoptosis in 15.9% of HeLa-S3 cells as compared with siNC-treated (7.7%) and PBS-treated (5.5%) HeLa-S3 cells. Infection of HeLa-S3 cells with H101 at a multiplicity of infection of 100 induced cell apoptosis (10.8%).

Next, we examined whether there was an additive effect on apoptosis when siBCL2 treatment was combined with H101. We found that the degree of apoptosis nearly tripled (32.5%) when both siBCL2 and H101 were administered together (Figure 3b). These data suggest that H101 synergizes with *Bcl2* siRNA in the induction of apoptosis.

Bcl2 siRNA enhanced H101 DNA replication

We then tested whether the apoptosis induced by *Bcl2* siRNA would interfere with viral replication of H101 and thus reduce the therapeutic effect of the oncolytic adenovirus if the two treatments were used together. To examine adenovirus DNA replication, the expression of the late gene *hexon* was determined by

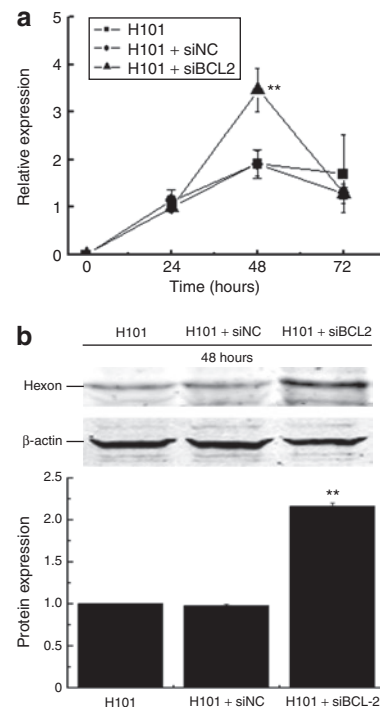


Figure 4 The effect of siBCL2 on viral DNA replication in HeLa-S3 cells. (a) Viral DNA replication was determined by real-time RT-PCR quantification of adenoviral late Hexon gene expression at 24, 48, and 72 hours after co-treatment, respectively. For comparison, the mRNA expression of Hexon at 24 hours in H101 group was arbitrarily set as 1, and β -actin was used as the internal control in the calculation. $P < 0.01$; compared to Hexon mRNA expression of the H101 group. (b) Western Blot analysis of Hexon protein expression at 48 hours. Bar: average band density of quantified Hexon protein after normalization by the internal control β -actin. Protein expression of Hexon in H101 group was arbitrarily set as 1. ** $P < 0.01$; relative to Hexon protein expression in the H101-treated group.

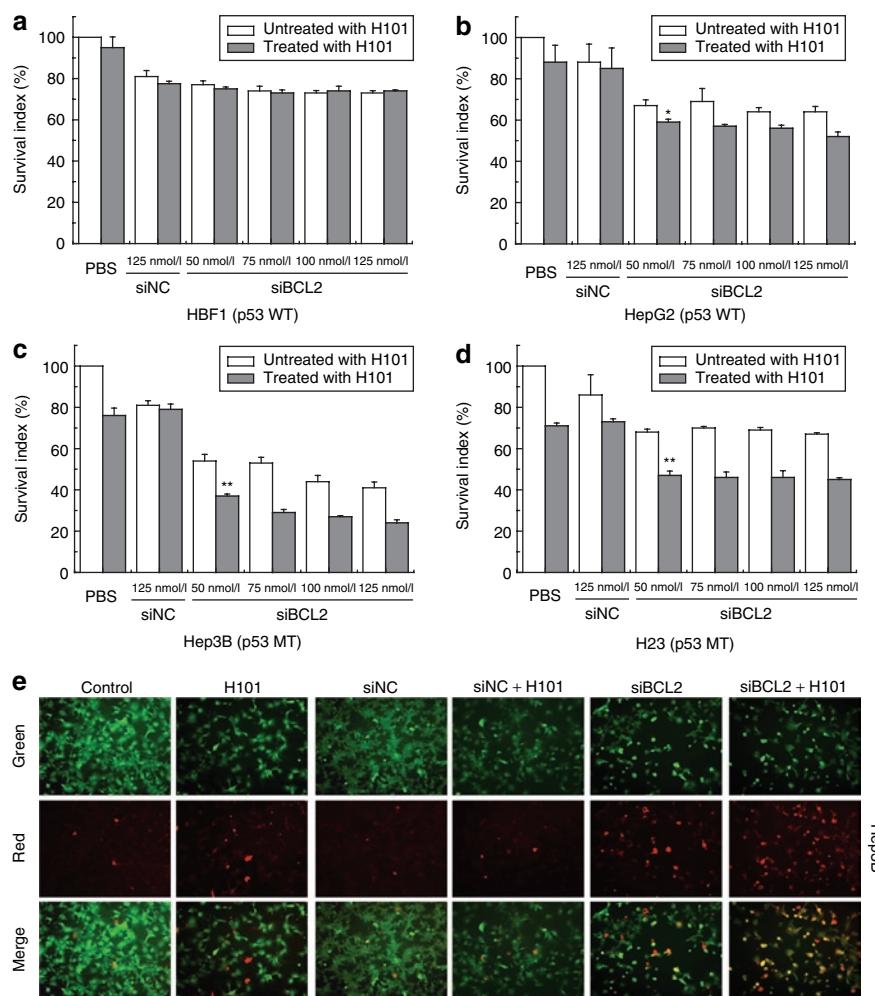


Figure 5 Growth inhibition of the combined therapy on human cell lines with varying p53 activity. **(a)** HBF1, normal human skin-derived fibroblasts; **(b)** HepG2, p53-wild-type hepatoma cell line; **(c)** Hep3B, p53-mutated hepatoma cell line; and **(d)** H23, p53-mutated non-small-cell carcinoma cell line. Cells were exposed to H101 (multiplicity of infection (MOI) = 100) and varying concentrations of siBCL2, and cell growth was measured by MTT assays. All data are presented as means \pm SD. of three independent experiments. **(e)** Cell apoptosis detected by TUNEL assay. Live cells were stained only with 6-CF (green) and necrotic cells with AnnCy3 (red). Apoptotic cells were stained with both AnnCy3 and 6-CF (yellow).

real-time RT-PCR. We did not observe significant differences in *hexon* mRNA expression in H101-infected cells in the presence or absence of siNC transfection. However, we found that siBCL2 treatment increased *hexon* mRNA expression by twofold in H101-infected cells at 48 hours ($P < 0.01$, **Figure 4a**). Similarly, western blot analysis also showed an approximately twofold increase in Hexon protein when the combination of siBCL2 and H101 were used (**Figure 4b**). Taken together, these data indicate that silencing of *Bcl2* actually enhanced DNA synthesis of H101, although the specific mechanism is not clear.

Enhanced cytotoxicity of the cocktail treatment in tumor cells with varied p53 activity

The sensitivity of cancer cells to oncolytic virus is affected by the activity of tumor suppressor p53. We thus tested the combined therapy in four cell lines with varying p53 activity, including HBF1 (normal human fibroblasts), HepG2 (hepatoma cell line with wild-type p53), Hep3B (hepatoma cell line with a p53 deletion^{25,26}), and H23 (non-small cell lung cancer cell line with p53 mutation²⁷). As

seen in **Figure 5a–d**, H101 had minimal effect on cell growth in normal fibroblasts. However, enhanced cytotoxic effects were observed in tumor cells, especially in Hep3B and H23 that bear p53 mutations. Similarly, an enhanced cell apoptosis, as determined by the TUNEL assay, was also observed in p53-negative Hep3B cells that were treated with the H101 and siBCL2 cocktail therapy (**Figure 5e**).

Improved *in vivo* antitumor activity by the combined treatment of siBCL2 and H101

To examine whether the enhanced *in vitro* cytotoxicity could be translated into *in vivo* animal testing, HeLa-S3 tumor cells were implanted in nude mice. When the volume of the xenografts reached 100–125 mm³, animals were treated by intratumor injection of siBCL2 and H101. As compared with the PBS control group, monotreatment with either oncolytic adenovirus H101 or siBCL2 inhibited tumor growth at a moderate rate (**Figure 6a**). However, tumor growth was significantly reduced in those animals that received the combined treatment of siBCL2 and H101 group ($n = 5$, $*P < 0.05$) in comparison with that in H101, siBCL2, or PBS treatment alone.

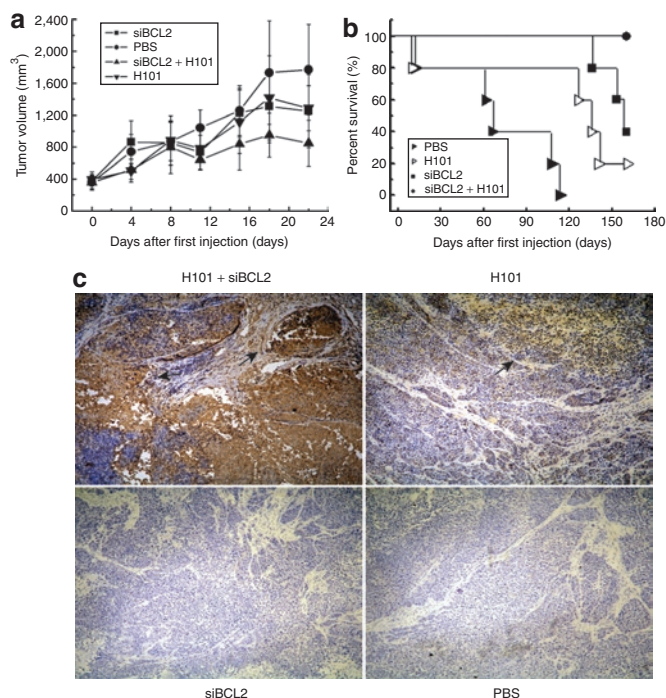


Figure 6 Antitumor activity of the cocktail treatment of siBCL2 and H101 in HeLa-S3 xenograft models. **(a)** Average volume of subcutaneous tumors after treatment with H101 (inverted triangle), H101 plus siBCL2 (triangle), siBCL2 (square), or PBS (circle). Values represent the means \pm SD for five animals per group. **(b)** Percentage of animal survival. Survival was evaluated over a period of 160 days. **(c)** Viral replication in tumors following the siBCL2 and H101 combined treatment. The tumor spread of adenovirus type 5 particles was determined by immunohistochemical detection using anti-adenoviral particle antibody. Original magnification: $\times 200$.

The long-term therapeutic effect of the combined treatment was examined by measuring animal survival in each treatment group. We observed an increase in survival in the group treated with the combined therapy (**Figure 6b**). By 160 days following treatment, all animals in the siBCL2/H101 group were alive and relatively healthy. In contrast, only 40% of animals that received siBCL2 monotherapy and only 20% of the animals that received H101 monotherapy were still alive. All of the animals in PBS control group died by day 120. At the end of the study, two of five mice that received siBCL2/H101 combined therapy had complete eradication of the pre-established tumors. These findings demonstrate that the combination of siBCL2 and H101 injection produced markedly improved antitumor outcomes *in vivo*.

To determine whether the increased production of adenovirus particles by siBCL as observed *in vitro* also occurred in this *in vivo* study, tumor tissues were collected and stained for adenovirus type 5 particle. As seen in **Figure 6c**, more adenovirus particles were present in tumors that were co-treated with siBCL2 and H101 than that in H101 and siBCL2 monotherapy, or PBS control.

DISCUSSION

Malignant tumors are associated with abnormalities in gene expression derived from both genetic and epigenetic lesions, including *p53* mutations²⁸ and *Bcl2* overexpression.²⁹ Targeting these tumor-specific abnormalities has become an increasingly

attractive area in developing molecular antitumor therapies,³⁰ in sharp contrast to traditional therapies that nonspecifically kill tumors as well as normal cells. One typical example is H101, a recombinant *E1B*, *E3*-deleted serotype 5 adenoviral drug, that specifically lyses tumor cells by targeting inactivated *p53*. As H101 adenovirus cannot replicate in normal cells because of the suppressive effect of wild-type *p53*, the virus should act selectively on tumor cells with mutated *p53* and spare normal cells.⁹

However, H101 has limited potential to eradicate tumors when used as monotherapy. Thus, H101 is often used in combination with traditional modalities, such as chemotherapy. In this communication, we studied the antitumor efficacy of H101 in conjunction with siRNA to *Bcl2*.

Bcl2 can be silenced by RNA antisense,^{30,31} DNA-targeted methylation,²³ and RNAi.^{10,21,22,32} To achieve more complete suppression of *Bcl2* expression, many studies chose plasmid-expressing shRNA or virus-expressing shRNA to knock down the target gene.^{33,34} Adenovirus vectors have been widely used as vehicles to deliver shRNA for gene silencing by virtue of their proven clinical safety. However, expression of adenoviral genes, virus-associated RNAi and virus-associated RNAII, resulted in the inhibition of RNAi activity, possibly through competitively interfering with Dicer or RISC activity.³⁵ Moreover, Pan *et al.* also found that oncolytic adenovirus vectors may not be efficient for long-term siRNA delivery.³⁶

We thus chose to downregulate *Bcl2* using chemosynthetic siRNAs. Synthetic *Bcl2* siRNA, although vulnerable to degradation when systematically administered, is relatively stable when injected directly into tumors. The H101 oncolytic adenovirus has been approved primarily for tumor topical injection in China. Thus, it would be feasible for the *Bcl2* siRNA oligonucleotide to be formulated together with H101 in a single intratumor injection solution, or co-administered locally as separate formulations.

Recently, we demonstrated that *Bcl2* siRNA can be efficiently expressed by incorporating siRNA oligonucleotide into the 3' untranslated region (3' UTR) of a tumor target gene, like TRAIL (J. Zhang, S. Huang, S. Ge, G. Qian, A.R. Hoffman, J.F. Hu, unpublished data). H101 oncolytic virus, although *E1B* and *E3*-deleted, has an intact *E1A* gene, permitting viral replication. It is therefore possible that *Bcl2* siRNA can be incorporated into the 3' UTR of the *E1A* of the adenovirus, leading to co-expression of *Bcl2* siRNA with the viral replication of H101 in tumors. Using this strategy, we would be able to combine the cocktail therapy as a single therapeutic agent applicable to clinics.

As demonstrated in **Figure 1**, siBCL2 led to efficient inhibition of *Bcl2* mRNA and protein expression. The RNAi activity was not inhibited in the presence of adenovirus H101. The combined action of *Bcl2* knockdown and H101 oncolysis effectively inhibited tumor growth, indicating that *Bcl2* knockdown can increase the therapeutic efficacy of H101.

In the animal studies, we used consecutive intratumoral injections of siBCL2 to overcome the short half-life of chemosynthetic siRNAs in animal models. The combined approach of H101 with siBCL2 showed superior tumor inhibition and prolonged survival compared with monotherapy with either agent alone. The enhanced antitumor effects of the combined approach in xenograft studies were consistent with the efficacy of co-treatment *in vitro*.

The precise mechanism of this enhanced activity of oncogene knockdown and viral oncolysis is still unclear. However, we found increased amplification of the adenovirus late gene *Hexon*, which encodes an adenovirus envelope protein, following *Bcl2* knockdown. It is possible that lysis of the tumor cells may release more viral progeny that can infect nearby tumor cells, allowing spread of adenoviral infection. In support with this explanation, we also observed enhanced spread of adenovirus particles in tumors by immunohistochemical analysis (Figure 6b). Thus, enhanced adenovirus oncolysis may partially explain the increased antitumor efficacy in our combined therapeutic approach. Recently, Yoon *et al.*³⁷ also demonstrated that the chemotherapeutic drug cisplatin significantly enhanced the number of adenovirus particles in tumor tissues. Similarly, Abou El Hassan *et al.*³⁸ also showed that paclitaxel increased viral gene expression in infected tumors. A similar mechanism may underlie the enhanced viral replication observed in this study.

It is also interesting to note that unlike the *in vivo* tumor studies, the enhanced viral replication induced by siBCL2 was only observed at 48 hours in the transfected cells (Figure 4a). The pattern of increased replication of H101 oncolytic virus disappeared after a longer period of incubation. The reason for this disparity between *in vitro* and *in vivo* studies is not clear, and may reflect the difference in dynamics of cell division and viral replication between *in vitro* two-dimensional cell culture and three-dimensional tumors. In cell culture, the adenovirus had a peak of replication at 48 hours following cell infection. Thereafter, viral replication slowed, probably as a consequence of cell death. A similar pattern of adenoviral replication was also observed by Abou El Hassan *et al.* in a non-small-cell lung cancer NCI-H460 cell line.³⁸

Theoretically, the replication of an oncolytic adenovirus should be restricted to tumor cells that are p53 deficient. However, it has been demonstrated that the E1B-deleted adenovirus ONYX-015 also replicated in some p53-positive tumor cells, but not in normal primary cells.³⁹ Recently, Rothmann *et al.* showed that the oncolytic virus was also able to replicate and kill primary human cells, including normal fibroblasts, keratinocytes, and mammary epithelial cells.¹¹ In this study, we also observed some therapeutic effect of the combined treatment of H101 and siBCL2 in p53-positive HepG2 hepatoma cells (Figure 5b), but this effect was still less effective than that seen in p53-defective Hep3B (Figure 5c) and H23 (Figure 5d) tumor cells. In agreement with Heise *et al.*,³⁹ we did not observe any cytopathic effects on normal fibroblasts (Figure 5a). Many factors may affect the cytopathic effect of the oncolytic adenovirus, including cell proliferation status before viral infection, cell-to-cell variation in viral response, and the purity and type of oncolytic adenovirus used.

As an apoptotic and/or survival “switch,” *Bcl2* plays a key role in the balance between proapoptotic and antiapoptotic factors in the intracellular microenvironment. Overexpression of *Bcl2* in tumors critically altered this balance and resulted in permanent survival of tumors. Thus, downregulating *Bcl2* may restore this balance and further increase the ability of tumor cells to respond to the apoptotic signal induced by exogenous stimuli. In this article, we observed enhanced apoptosis in combined treatment compared to monotreatments by either siBCL2 or by H101. Adenovirus-expressing proteins, such as E1A, activate the cellular

apoptotic pathway.^{40,41} Induction of H101 viral replication may act as a potential apoptotic trigger inducing tumor apoptosis and synergizing with siBCL2, accounting for enhanced antitumor effects.

In conclusion, this study provides support for the combined use of an oncolytic adenovirus with *Bcl2* siRNA as a promising approach in cancer gene therapy, either formulated as a single intratumor injection solution, or co-administered as separate formulations. Alternatively, the oncolytic adenovirus can be further modified by integrating *Bcl2* siRNA into the H101 viral genome.

MATERIALS AND METHODS

Cell culture and recombinant Adenovirus H101. All human tumor cells were purchased from American Type Culture Collection (Manassas, VA), including Hela-S3 (cervical carcinoma), HepG2 (hepatoma), Hep3B (hepatoma), and H23 (non-small-cell lung carcinoma). HBF1 was a normal human fibroblast cell line cultured from human fetal skin.⁴² Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, CA) supplemented with 10% (vol/vol) fetal calf serum and maintained at 37°C in a 5% CO₂ atmosphere. Recombinant adenovirus H101 was kindly provided as a gift by Shanghai Sunway Biotech (Shanghai, China) and was maintained under conditions recommended by the manufacturer.

***Bcl2* siRNA oligonucleotide.** Two *Bcl2* siRNAs (siBCL2 and siBCL2-X) were designed from the same sequence locations of *Bcl2* as described previously.²² *Bcl2* siRNA oligonucleotides (siBCL2 and siBCL2-X, Figure 1a) and control siRNA (siNC: 5' UUCUCCGAACGUGUCACGU 3') were synthesized and purified by Shanghai Genephrama (Shanghai, China).

Co-treatment of tumor cells with *Bcl2* siRNA and oncolytic adenovirus H101. Hela-S3 cells at 30–50% confluence in 6-well plates were transfected with 50 nmol/l siBCL2 or siNC using a Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA). After overnight incubation, cells were infected with H101 at a multiplicity of infection of 100. Control groups included cells that were transfected with siBCL2, siNC, or PBS.

RNA extraction and reverse transcription-PCR analysis. Total RNA was isolated from cells using Trizol reagent (Invitrogen) following the protocol provided by the manufacturer. First-strand cDNA was synthesized with RNA reverse transcriptase as described.^{43,44} Briefly, 1 µg of total RNA was used, and PCR was carried out in 50 µl reaction containing 5 µl of 10× PCR buffer, 1 µl of 10 mmol/l dNTP Mix, 1 µl of 10 µmol/l each primer, 1.5 U of *Taq* DNA polymerase, and 2 µl of sample cDNA. After denaturation at 95°C for 5 minutes, *Bcl2* cDNA was amplified by 35 cycling regimen consisting of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s, and subsequently with the extension at 72°C for 10 minutes. Primers used for amplifying *Bcl2* and β -actin mRNA were as follows: *Bcl2* sense: 5'-ATGTGTGTGGAGAGCGTCAA-3', and *Bcl2* antisense: 5'-CAGGAGAAATCAA ACAGAGGC-3'; β -actin sense: 5'-CCAAGGCCAACCCGCGAGAAGATGAC-3', and β -actin antisense: 5'-AGGGTACATGGTGGTCCGCCAGAC-3'. Amplified PCR products of the expected size were quantified by densitometric measurements and normalized to β -actin values.

Quantitative real-time RT-PCR amplification was carried out using QuantiTect SYBR green (Qiagen, Valencia, CA) as previously described.⁴² Specifically, total RNA was extracted by Trizol reagent (Invitrogen), and cDNA was synthesized with RNA reverse transcriptase. The C_T (threshold cycle) value of *Hexon* was quantitated by Q-PCR in triplicate using an ABI Prism 7900 HT sequence detector (AB Applied Biosciences, CA) following the manufacturer's protocol and was normalized over the C_T of the β -actin control.

MTT assay. Cells were seeded at 5,000 cells per well in flat-bottomed 96-well plates. At the end of the incubation time, 20 µl of 5 mg/ml MTT

(Sigma, St Louis, MO) in PBS was added to each well. After 4 hours, media were discarded, and cells were lysed with 100 μ l dimethylsulfoxide. Cells were incubated for a further 30 minutes at 37°C with gentle shaking. The optical density was determined with a microplate reader at 570 nm. Absorbance values in the treated groups were normalized to the values of untreated tumor cells to calculate the percentage of survival.

Western blot analysis. *Bcl2* and viral Hexon proteins were quantitated by western blotting as previously described.⁴⁵ Cells were harvested at the indicated time, and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 12% (wt/vol) polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were incubated with secondary antibody conjugated to a fluorescent tag; the bands were visualized and quantified by Odyssey infrared Imaging System (LI-COR, Lincoln, NE). The following antibodies were used: *Bcl2* (Santa Cruz Biotechnologies, Santa Cruz, CA), Hexon (Abcam, Cambridge, UK), and β -actin (Santa Cruz Biotechnologies).

Apoptotic analysis. Apoptosis was determined by dual staining with annexin-V-fluorescein isothiocyanate and propidium iodide and analyzed by flow cytometry. Cells were prepared according to the manufacturer's instruction provided in the Annexin V-FITC apoptosis kit (BD Biosciences, San Diego, CA). Apoptosis was quantified on a fluorescence-activated cell sorter (Becton Dickinson, Sunnyvale, CA), and data from 10,000 events were collected for further analysis.

Apoptotic cells were also stained with Annexin V-CY3 apoptosis detection kit (Sigma). Briefly, tumor cells were labeled with 6-carboxy fluorescein (6-CF) and Annexin V-Cy3 (AnnCy3) at room temperature and were observed by fluorescence microscopy. Live cells were stained only with 6-CF (green), whereas necrotic cells stained only with AnnCy3 (red). Cells starting the apoptotic process were stained with both AnnCy3 and 6-CF.

Tumor xenograft model in nude mice. HeLa-S3 cell tumor xenografts were established by s.c. injection of 5×10^6 cells into the right flank of 4–6-week-old female athymic nude mice. When tumors reached the required mean tumor volume (100–125 mm³) as determined by the formula volume = length \times width² \times 0.5, animals were randomly assigned into four groups. The siBCL2 plus H101 group received intratumoral injections of 10 μ g siBCL2 on day 1, 4, 8, 11, 15, and H101 adenovirus at 1×10^8 plaque-forming units on day 2, 5, 9, 12, 16; the siBCL2 group received five intratumoral injections of 10 μ g siBCL2; H101 adenovirus group received five intratumoral injections of H101; control group mice received five injections with PBS. Non-target control RNAi oligos, including siNC, had been extensively examined in our previous studies^{22,46,47} and were not included in this animal study. The tumor size was measured by vernier calipers every 4 days. Animal experiments were performed in accordance with institutional guidelines for animal care by Jiao Tong University.

Immunohistochemical analysis. Mice from each group were selected randomly and killed on day 7 after treatment for immunohistochemical staining.⁴⁸ Tumor samples were taken, fixed in 10% formalin, embedded in paraffin, and 3- μ m sections were cut. Tumor sections were incubated at 4°C overnight with a mouse anti-adenovirus type 5 antibody [M58] (Abcam, Boston, MA) at a dilution of 1:50. Sections were rinsed in PBS-T (0.05% Triton X-100 in PBS), followed by the biotinylated rat anti-mouse secondary antibody at a 1:500 dilution for 1 hour at room temperature. After washing twice with PBS-T, slides were subsequently incubated with streptavidin-horseradish peroxidase (BD Biosciences) and diaminobenzidine substrate to develop the colorimetric reaction. All slides were counterstained with hematoxylin.

Statistical analysis. All experiments were performed in triplicate, and the data were expressed as mean \pm SD. The comparative C_T method was applied in the quantitative real-time RT-PCR assay according to the delta-delta C_T

method. The data were analyzed with Student's *t*-test or by one-way analysis of variance, and results were considered statistically significant at $P \leq 0.05$.

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