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Adenovirus-mediated small hairpin RNA targeting Bcl-XL as therapy for colon cancer

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Bcl-XL, an anti-apoptotic protein of Bcl-2 family, is overexpressed in colon cancers. To determine Bcl-XL's potential feasibility as a therapeutic target, we constructed a recombinant adenovirus that expressed a U6 promoter-driven small hairpin RNA (shRNA) targeting Bcl-XL (Ad/Bcl-XL shRNA) and evaluated the vector's ability to induce RNA interference *in vivo* and alter apoptosis induction in colon cancer cells and tumours. Ad/Bcl-XL shRNA effectively knocked down Bcl-XL expression in colon cancer cells and decreased their viability. Treatment with Ad/Bcl-XL shRNA but not control vectors led to dramatically increased cleavage of cellular apoptosis-related enzymes caspase-9, caspase-3 and poly (ADP-ribose) polymerase. Ad/Bcl-XL shRNA also significantly suppressed the growth of subcutaneous tumours derived from DLD1 cells in a nude mouse model and did so without causing any obvious damage to normal tissues or normal human fibroblasts. Together, our results support the feasibility of using adenovirus-mediated RNA interference therapy targeting Bcl-XL against colon cancers and warrant further studies of its safety and efficacy.

Key words: adenovirus; Bcl-XL; RNA interference; small hairpin RNA; colon cancer

Apoptosis can be initiated extrinsically (*via* receptor pathways), intrinsically (*via* mitochondrial pathways), or both.[1] Death signals from death receptors may be transduced to mitochondria by Bcl-2 family proteins, which subsequently trigger the release of cytochrome c and initiate the apoptosome amplification loop.[1,2] Thus, Bcl-2 family members are critical for apoptosis induction. Unbalanced expression of Bcl-2 proteins impairs apoptotic signaling pathways. For example, overexpression of the anti-apoptotic protein Bcl-XL can lead result in a multiple drug-resistant phenotype.[3] On the other hand, as has been shown in various cancer cells, enforced expression of the pro-apoptotic Bax protein can induce apoptosis.[4–7]

A growing body of evidence suggests that the ratio of Bcl-2 to Bax is an important indicator of resistance to apoptosis.[7–9] Recent studies have shown that expression of Bcl-2 is low and that of Bcl-XL is high in colon cancer.[10,11] In our own laboratory, we have shown that downregulation of Bcl-XL by small interfering RNA (siRNA) can induce apoptosis in 5-fluorouracil-resistant colon cancer cells and overcome the acquired resistance to tumour necrosis factor-related apoptotic inducing ligand (TRAIL) protein in DLD1 cells.[12,13] Thus, Bcl-XL represents a potential new therapeutic target in colon cancer.

The therapeutic effect of RNA interference depends on the stability and tissue specificity of siRNA and the efficiency of siRNA transduction. Recent studies have shown that using DNA plasmids to express double-stranded RNA can increase siRNA stability.[14,15] However, the efficiency of plasmid delivery *in vivo* remains poor. Therefore, more efficient means of delivering therapeutic siRNA *in vivo* are necessary. One possibility is the use of adenoviral vectors, which can efficiently transduce genes of interest into a broad range of cell types and have been used extensively as gene delivery vehicles. Therefore, in the present study, we set out to determine whether a recombinant adenovirus specifically designed to express a U6 promoter-driven small hairpin RNA (shRNA) targeting Bcl-XL could induce RNA interference and whether its expression would have any therapeutic effects on colon cancer *in vivo*.

Material and methods

Cells and cell culture

Human colon cancer cell lines DLD-1 and LOVO (Institute of Cell Research, Shanghai, China) and normal human fibroblast (NHFB) cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% glutamine and a 1× antibiotic–antimycotic mixture (Invitrogen, Beijing, China). All cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

Tissue samples

Tumour tissue specimens were obtained intraoperatively from 40 consecutive patients who underwent surgical resection of primary sporadic colorectal adenocarcinomas at Sir Run Run Shaw Hospital (Hangzhou, China) between November 2004 and April 2005. In each case, tissue specimens were also obtained from areas adjacent to but >5 cm away from the cancerous tissue. None of the patients had received any preoperative chemotherapy or radiotherapy before surgery. All patients gave their informed consent to participate in the study before undergoing tissue resection.

Reverse transcription-polymerase chain reaction analysis

In the case of tissue specimens, a 100-μg sample of each specimen was prepared for RT-PCR analysis by homogenizing it together with liquid nitrogen in a grinder and lysing the homogenate in TRIzol reagent (Invitrogen, Beijing, China). In the case of cultured cells, samples were directly lysed in TRIzol reagent after treatment with adenoviral shRNA. In either case, RNA was extracted according to Invitrogen's recommended protocol. Next, 1 μg of RNA from each sample was incubated with random hexamers (Applied Biosystems, Shanghai, China), which served as primers to drive the reverse transcription of RNA into cDNA. Then, typical PCR was performed to amplify any *bcl-2, bcl-xl* and *bax* genes present in a sample. The human *GAPDH* gene was used as an internal control for mRNA loading and normalization. The sequences of primers were as follows: 5′-GGTGCCACCTGTG

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GTCCACCTG-3′ and 5′-GTTCACTGTTGGCCCAGATAGG-3′ for *bcl-2*; 5′-GTGAATGGAGCCACTGCGCA-3′ and 5′-CCC-CATCCCGGAAGAGTTCA-3′ for *bcl-xl*; 5′-GCCCACCA GC-TCTGAGCAGATCAT-3′ and 5′-CGGCAATCATCCTCTGCA-GC-3′ for *bax*; and 5′-ACCACAGTCCATGCC ATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for *GAPDH*.

Immunohistochemical staining

Immunohistochemical staining was done using a MaxVision kit (Maixin Biol, Fuzhou, China). In brief, 4-μm-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks; mounted on polylysine-coated slides; dewaxed in xylene; and rehydrated through a graded series of ethanol. After deparaffinization, slides were treated with 3% hydrogen peroxide in methanol solution for 10 min to quench endogenous peroxidase activity and then autoclaved at 121°C for 5 min in 10 mM sodium citrate buffer (pH 7.4) to facilitate antigen retrieval. Nonspecific binding reactions were blocked by treating the slides with 10% normal goat serum for 10 min. Thereafter, the slides were incubated at 4°C overnight with primary antibodies specific for Bcl-2 (Santa Cruz, CA, 1:100 dilution), Bcl-XL (Dako, Glostrup, Denmark, 1:100 dilution) and Bax (Santa Cruz, CA, 1:100 dilution), respectively. Next, 1 drop of MaxVision™ reagent was applied to each slide, after which the slides were incubated for 30 min at room temperature. Colors indicative of staining were developed by incubating the slides in a solution of 0.05% diaminobenzidine and 0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) for 5 min. Finally, the slides were counterstained with 1% Meyer's hematoxylin. As a negative control for Bcl-2, Bcl-XL and Bax staining, tissue samples were also treated with normal serum instead of each primary antibody.

Evaluation of immunohistochemical staining

All tissue specimens were scored blindly under a light microscope. Cells exhibiting a clear structure and brown–yellow granules were considered positive; all others were considered negative. Five to ten high-magnification fields (400×) were examined on each slide. Percentage of positive cell was scored on a scale of 0–4 as follows: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). Staining intensity was scored on a scale of 1–3 as follows: 1 (yellow), 2 (brown–yellow) and 3 (brown). Finally, the scores for percent positive cells and staining intensity were combined and ranked as follows: – (0–1), + (2–3), ++ (4–5) and +++ (≥6).[16]

Recombinant adenovirus construction

An adenoviral vector containing Bcl-XL shRNA and a U6 promoter to drive the expression of Bcl-XL-targeting siRNA sequences (Ad/Bcl-XL shRNA) was constructed as described previously.[12,13] The Bcl-XL siRNA in the vector was connected by a hairpin structure formed by annealing a pair of oligonucleotides termed Bcl-XL sense (3′-GGAGATGCAGGTATTGGTGGTA-CGTAGGCACCAATACCTGCATCTCCCTTTTTG-5′) and Bcl-XL Eco (3′-AATTCAAAAAGGGAGATGCAGGTATT GGTG-CCTACGTACCACCAATACCTGCATCTCC-5′). The resulting shRNA, which contained an SnaBI digestion site in the middle of its sequence and and EcoRI digestion site on its 3′ end, was then cloned into a pBabe-U6 plasmid by ApLI and EcoRI enzymes to obtain a new plasmid named pBabe-U6-Bcl-XL shRNA and then inserted into a shuttle plasmid and recombined with an adenoviral backbone in 293 packaging cells. A control adenoviral vector having the same adenoviral backbone but containing a cytomegalovirus promoter to drive expression of a green fluorescent protein gene (Ad/CMV-GFP) was also constructed as described previously.[6] The newly constructed vectors were then amplified in 293 packaging cells and purified by CsCl ultracentrifugation. Finally, their titers were determined by optical absorbance at a wavelength of 260 nm (one A_{260} unit = 10^{12} particles/mL) and by plaque assay. All of the resulting viral preparations were determined to be free of contamination by wild-type adenovirus and endotoxin.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT used was purchased from Sangon (Shanghai, China). In brief, cells were first seeded in 96-well plates at concentrations of 5×10^3 cells/well, incubated for 1 day and then treated with different doses of adenoviruses for specific periods of time ranging from 24 to 96 hr. Then, 100 μL of MTT solution (10 mg/mL) was added to each well, and the cells were incubated for an additional 4 hr, after which the medium was removed. Next, 100 μL of dimethyl sulfoxide was added to each well and the cells were incubated for 10 min more. Finally, cell viability in each well was measured in terms of optical density at a wavelength of 570 nm (OD_{570nm}). All results were normalized against the OD_{570nm} for phosphate-buffered saline (PBS)-treated cells to determine relative levels of cell viability. Each cell viability assay was performed in quadruplicate and repeated at least twice.

Cell clonogenicity assay

Cell clonogenicity was assayed as follows. In brief, 1×10^3 cells were placed in normal culture medium in 10-cm dishes and incubated overnight. Then, the cells were treated with Ad/Bcl-XL shRNA or Ad/CMV-GFP at multiplicities of infection (MOIs) (*i.e.*, viral particles/cell) ranging from 500 to 2,000 MOI or with PBS alone (mock treatment control). Then, the cells were cultured in an incubator containing 5% CO_2 at 37°C for 14 days more. Next, individual colonies (>50 cells/colony) were fixed and stained with a solution containing 0.25% crystal violet and 20% ethanol for 20 min. The colonies were counted with Optimas software (Media Cybernetics LP, Silver Spring, MD). Each experiment was done in triplicate and repeated at least twice.

Western blot analysis

To evaluate the potential mechanisms of Ad/Bcl-XL shRNA's cell-killing effect on colon cancer cells, the expression of apoptosis-related proteins Bax, Bcl-XL, Caspase-3, Caspase-9 and poly (ADP-ribose) polymerase (PARP) was evaluated by western blot analysis. In brief, DLD1 cells that had been treated with Ad/Bcl-XL shRNA for 72 hr were collected, washed with cold PBS, and then lysed in Laemmli's lysis buffer. Then, equal amounts of the resulting lysate were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Hybond enhanced chemiluminescence (ECL) membranes (Amersham Bioscience, Buckinghamshire, England). The membranes were then blocked with PBS buffer containing 5% low fat milk and 0.05% Tween 20 for 1 hr or overnight at 4°C, washed 3 times with PBS containing 0.05% Tween 20 (PBST) and incubated with primary antibodies for at least 1 hr at room temperature. After washing with PBST again, the membranes were incubated with peroxidase-conjugated secondary antibodies and developed with an ECL detection kit (Amersham Bioscience, Buckinghamshire, England). Rabbit anti-human Bax, Bcl-XL, Caspase-3 and Caspase-9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human PARP antibody was obtained from PharMingen (San Diego, CA). β-actin was used as a loading control.

Analysis of subcutaneous tumour growth in vivo *in mice*

To determine whether Ad/Bcl-XL shRNA could effectively suppress tumour growth *in vivo*, subcutaneous tumours were established in 6- to 8-week-old nude mice (Institution of Animal Research, Shanghai, China) by injecting 4×10^6 DLD1 cells into the dorsal flank of each mouse. After growing to 3–5 mm in diameter, the tumours were injected Ad/Bcl-XL shRNA, Ad/GFP (vector control), or PBS (mock treatment control) at a dosage of 3×10^{10} viral particles 3 times a week for 1 week. Each treatment

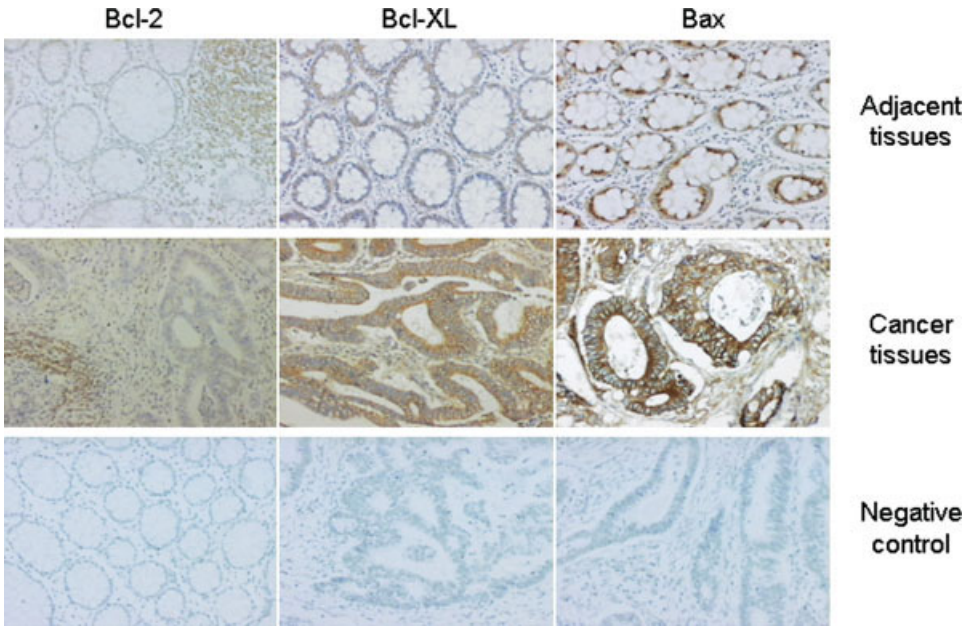

FIGURE 1 – Immunohistochemical analysis of *Bcl-2*, *Bcl-XL* and *Bax* gene expression in tumour and adjacent tissue samples from patients with colorectal cancer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I – PATIENT'S DISTRIBUTION BY RATIOS OF MRNA AND PROTEIN EXPRESSION IN ADJACENT TISSUES *VS.* TUMOR FOR BCL-XL, BCL-2, AND BAX (TOTAL PATIENTS: 40)

Target molecule	Patient's distribution by ratios of expression in adjacent tissue *vs.* tumour			*p* value
	Patients with ratio >1	Patients with ratio =1	Patients with ratio <1	
Bcl-XL				
mRNA	6 (15)	3 (7.5)	31 (77.5)	0.01
protein	5 (12.5)	13 (32.5)	22 (55)	0.02
Bcl-2				
mRNA	11 (27.5)	16 (40)	13 (32.5)	0.80
protein	7 (17.5)	19 (47.5)	14 (35)	0.09
Bax				
mRNA	12 (30)	2 (5)	26 (65)	0.06
protein	7 (17.5)	19 (47.5)	14 (35)	0.09

Values inside parenthesis are percentages.

group included 8 mice, which were treated and cared for in accordance with the institutional guidelines of Zhejiang University. Tumour volumes were monitored every 2 days for 1 month. The volumes were calculated using the formula $a \times b^2 \times 0.5$, where a and b represented the larger and smaller diameters, respectively. Mice were euthanized when their tumours grew to 1.5 cm in diameter. Tumours tissues or other organs were excised, sectioned, processed and subjected to hematoxylin and eosin staining in the department of pathology at Sir Run Run Shaw Hospital The toxicity of Ad/Bcl-XL shRNA was evaluated by collecting blood samples from the tail veins of 3 mice 2 days after the last adenoviral or mock treatment and analyzing them for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as previously described.[7]

Statistical analysis

Differences among the treatment groups were assessed by the Wilcoxon matched paired test or analysis of variance (ANOVA), using commercially available statistical software (StatSoft, Tulsa, OK). $p < 0.05$ was considered significant.

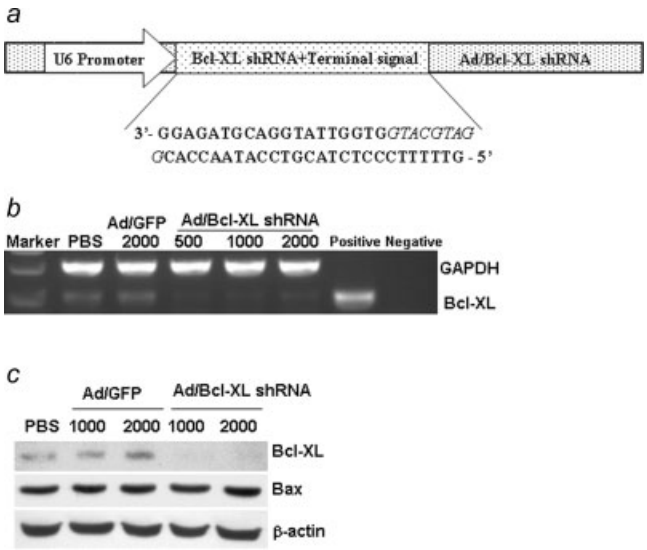

FIGURE 2 – Downregulation of Bcl-XL expression by Ad/Bcl-XL shRNA. (*a*) Shematic diagram of Ad/Bcl-XL shRNA. Beneath the diagram is the sequence of Bcl-XL shRNA including its terminal signal. (*b*) RT-PCR analysis. DLD1 cells were treated with different doses of Ad/Bcl-XL shRNA for 48 hr and then processed to extract mRNA for RT-PCR. (*c*) Western blot analysis. DLD1 cells were treated with various doses of Ad/Bcl-XL shRNA for 72 hr and then evaluated for Bcl-XL expression by western blot analysis. Ad/CMV-GFP was used as a vector control and PBS as a mock control. Data presented here are from 1 of 2 experiments that produced similar results.

Results

Bcl-2, Bcl-XL and Bax gene expression in tumour and adjacent tissue samples from patients with colorectal cancer

Bcl-2 expression at the mRNA and protein levels was undetectable or very low in most tissue samples. In contrast, *Bcl-XL* expression at both the mRNA and protein levels was moderately intense, and *Bax* expression was very intense (Fig. 1, mRNA data

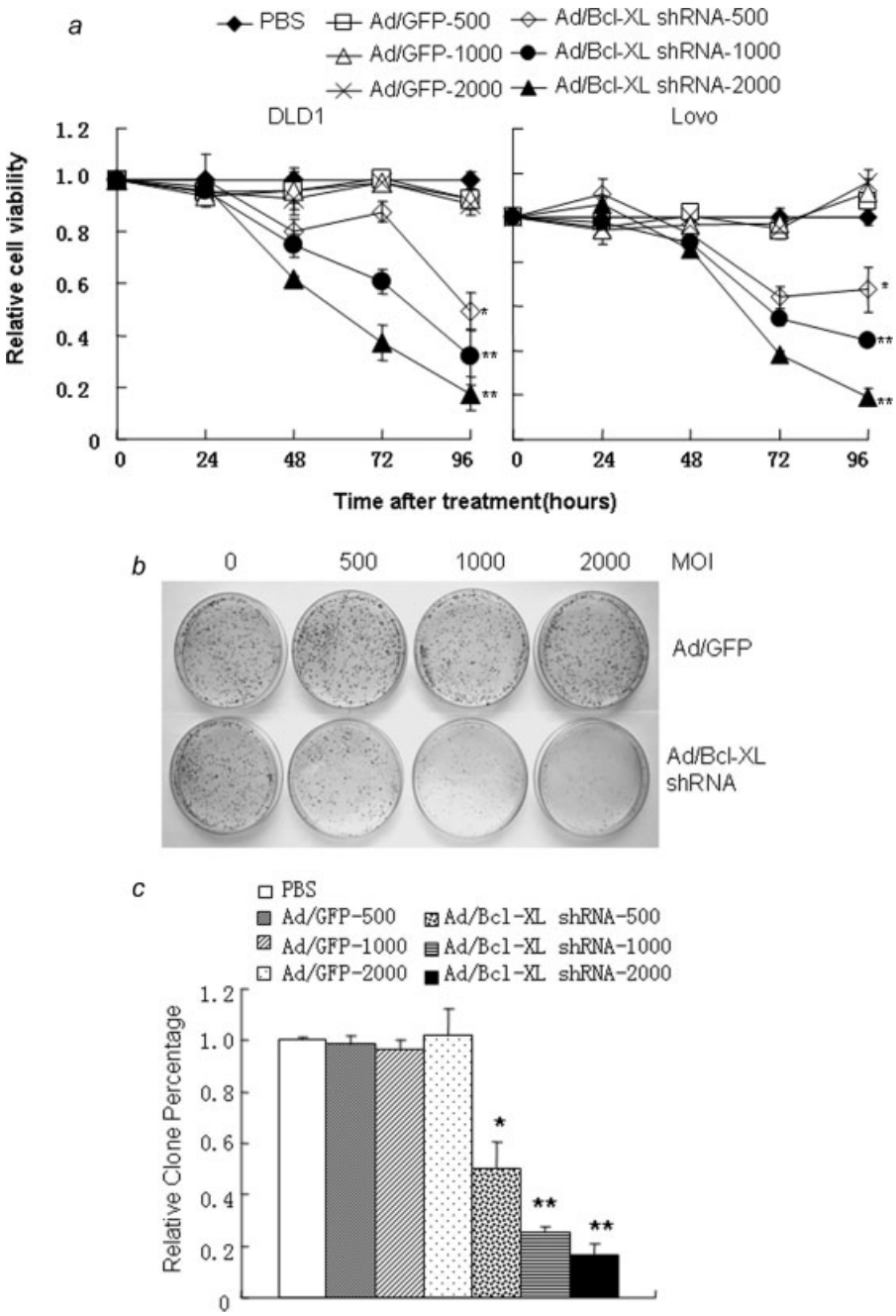

Figure 3 – Effects of Ad/Bcl-XL shRNA on colon cancer cells. (*a*) MTT assay. Cells were treated with various doses of adenovirus (MOIs). Ad/CMV-GFP was used as a vector control. Cell viability (mean ± SD) was determined at the indicated time points after treatment. $*p < 0.05$; $**p < 0.01$). (*b*) Clonogenicity assay. DLD1 cells were treated with various doses of adenovirus (MOIs) for 14 days. Ad/CMV-GFP was used as a vector control. Data presented here are from 1 of 3 experiments that produced similar data. (*c*) Quantification of data (mean ± SD) from 3 clonogenicity assays. $*p < 0.05$; $**p < 0.01$).

not shown). However, there were more individual patients with higher ratios of mRNA and protein expression in tumour *vs.* adjacent tissues for *Bcl-XL* than for *Bcl-2* or *Bax* ($p < 0.05$, Table I).

Ad/Bcl-XL shRNA-induced downregulation of Bcl-XL expression in colon cancer cells

Ad/Bcl-XL shRNA (Fig. 2*a*) effectively knocked down *Bcl-XL* mRNA expression levels in colon cancer cells even at the lowest dose of 500 MOI (Fig. 2*b*). Moreover, as shown by western blot analysis and comparison with mock-treated controls, Ad/Bcl-XL shRNA was also able to downregulate Bcl-XL protein expression at 72 hr after treatment regardless of dose, whereas the control vector (Ad/GFP) was not able to do so (Fig. 2*c*).

Cell-killing and clonogenic effects of Ad/Bcl-XL shRNA

Ad/Bcl-XL shRNA significantly decreased the viability of DLD1 and LOVO cells at 72 hr or more after treatment, whereas PBS or the control vector alone had almost no effect at all at the same time points ($p < 0.05$, Fig. 3*a*). Ad/Bcl-XL shRNA's cell-killing effect was also dose-dependent, being observed at all doses

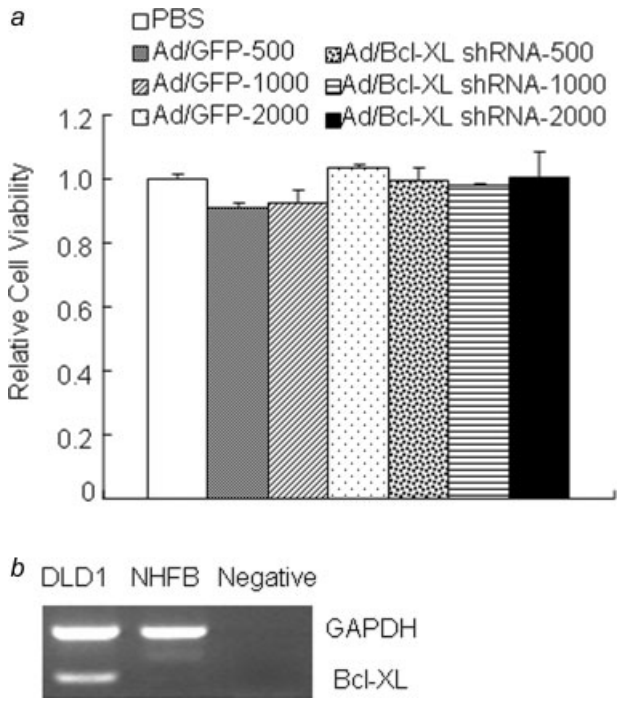

Figure 4 – Effects of Ad/Bcl-XL shRNA on normal human fibroblasts (NHFB). (*a*) Cell viability (mean ± SD) after treatment with Ad/Bcl-XL shRNA, was evaluated by MTT assay. (*b*) *Bcl-XL* mRNA expression levels in DLD1 and NHFB cells was analyzed by RT-PCR.

(including 500 MOI) but definitely increasing as dose increased (Fig. 3*a*). Moreover, treatment with Ad/Bcl-XL shRNA dramatically inhibited clonogenicity (*i.e.*, colony formation) of DLD1 cells, even at the lowest dose used (500 MOI) ($p < 0.05$, Figs. 3*b* and 3*c*). Again, this inhibition of colony formation was dose-dependent (Fig. 3*c*).

Important to note is that neither Ad/Bcl-XL shRNA nor control vector Ad/CMV-GFP exerted any obvious cell-killing effects on NHFB cells even after 96 hr and even at the highest dose given (2,000 MOI) (Fig. 4*a*). Moreover, as further analysis revealed, NHFB cells expressed significantly less *Bcl-XL* mRNA than did DLD1 cells (Fig. 4*b*), which might explain in part why the NHFB cells were relatively more resistant to Ad/Bcl-XL shRNA.

Cleavage of apoptotic signaling by Ad/Bcl-XL shRNA

As shown by western blot analysis, treatment with Ad/Bcl-XL shRNA led to increased cleavage of Caspase-9, Caspase-3 and PARP, whereas treatment with PBS or Ad/CMV-GFP alone had no such detectable effects (Fig. 5). This implied that the Ad/Bcl-XL shRNA-induced killing of colon cancer cells was mediated by the induction of apoptosis.

Tumour growth suppression by Ad/Bcl-XL shRNA in vivo

In the mice experiments *in vivo*, treatment with Ad/Bcl-XL shRNA dramatically and significantly suppressed the growth of subcutaneous tumours derived from DLD1 cells. This suppression was significant when compared to the effects of Ad/CMV-GFP and mock treatment on tumour growth (Fig. 6*a*, $p < 0.05$). Meanwhile, treatment with PBS on one hand and Ad/CMV-GFP on the other resulted in similar levels of tumour growth ($p > 0.05$). Mean serum liver transaminase levels on Day 2 after last treatment did not significantly differ among groups (Fig. 6*b*). Histopathologic analysis of heart, kidney, liver and lung tissues revealed no obvious damage because of the intratumoural administration of Ad/Bcl-XL shRNA (Fig. 6*c*). In addition, histopathologic examination of hematoxylin- and eosin-stained tissue sections revealed

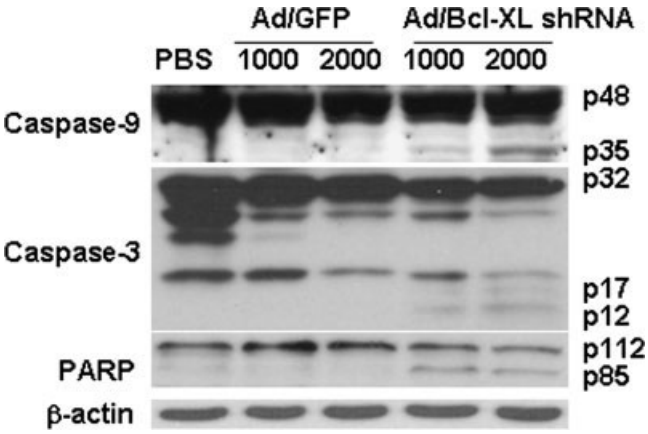

Figure 5 – Ad/Bcl-XL shRNA-activated cleavage of caspases and PARP. DLD1 cells were treated with various doses of Ad/Bcl-XL shRNA for 72 hr, harvested and subjected to western blot analysis. Data presented here are from 1 of 2 experiments that produced similar results.

no obvious inflammation at 3 days after the first injection in any of the 3 treatment groups (Fig. 6*c*).

Discussion

In our study, we found that the anti-apoptotic protein Bcl-XL, but not Bcl-2, to be more frequently overexpressed in colon cancer tissues than in adjacent tissues, thus making Bcl-XL a potentially useful therapeutic target in colon cancer. Pursuing this hypothesis, we evaluated the effects of adenovirally delivered Bcl-XL shRNA *in vitro* and *in vivo* and obtained very promising results. Ad/Bcl-XL not only effectively knocked down Bcl-XL expression and activated apoptotic signaling pathways in colon cancer cells, *in vitro*, but also significantly inhibited the viability of colon cancer cells *in vitro* and the growth of subcutaneous tumours in mice *in vivo*. Together, these findings confirmed the potential utility of Bcl-XL targeting and the feasibility of adenovirally mediated shRNA delivery in colon cancer therapy.

Because Bcl-XL is expressed in a wide variety of cells, including normal cells and plays an important role in embryonic development, it is possible that use of a constitutive U6 promoter to knock down Bcl-XL expression might have treatment-limiting toxic effects. Therefore, we evaluated the toxicity of Ad/Bcl-XL shRNA in NHFB cells. We found them to be resistant to the vector. Since we had also shown previously that NHFB cells are sensitive to infection with recombinant adenoviral vectors,[17] we therefore considered it unlikely that the resistance of NHFB cells seen in the present study was caused by resistance to the adenoviral vector itself. One possible alternative explanation is that Bcl-XL expression may not be critical to the survival of normal cells such as NHFB cells; we inferred this from our observation that, like normal tissues, NHFB cells expressed low levels of, *Bcl-XL* (Fig. 4*b*). Furthermore, we found no histopathologic evidence of obvious toxicity or damage caused by Ad/Bcl-XL shRNA administered intratumourally. Together, our data suggest that Ad/Bcl-XL shRNA can induce relatively tumour-specific cytotoxicity. They also corroborate previous demonstrations by others that RNA interference can be induced using DNA plasmids.[18,19] Thus, it appears feasible and safe to use adenoviruses, which are known for their high transduction efficiency in various mammalian cells and tissues, to deliver therapeutic shRNA molecules into tumour cells *in vivo*.

Yet, despite the extensive experimental and clinical experience with adenoviruses as gene transfer vehicles, their potential immunogenicity when administered systemically remains a major concern.[20] This was addressed in a recent study in mice, in which

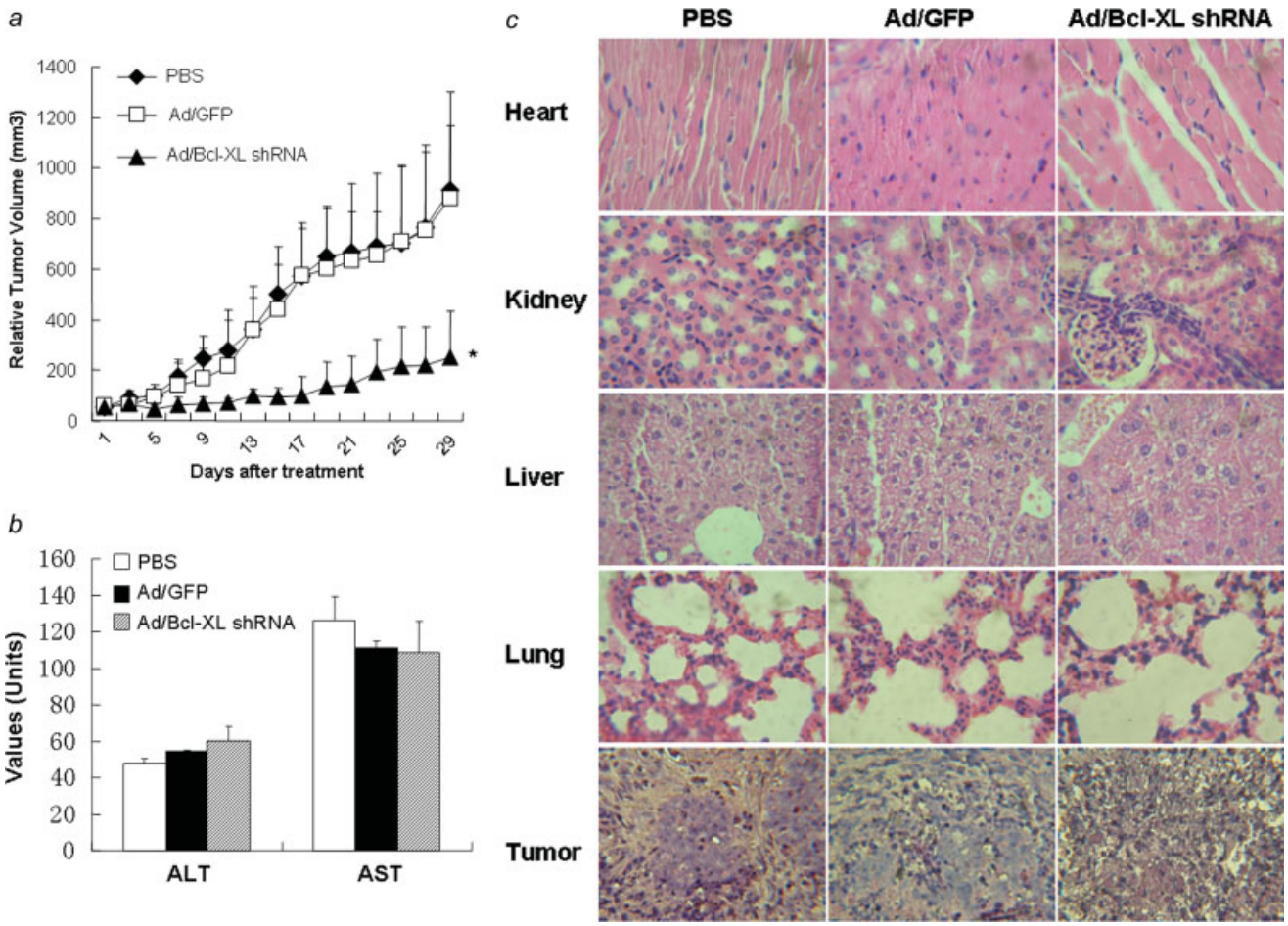

FIGURE 6 – Effects of Ad/Bcl-XL shRNA on a subcutaneous tumour model in mice. (*a*) Ad/Bcl-XL shRNA-induced suppression tumour growth *in vivo*. Subcutaneous tumours derived from DLD1 cells were treated with various agents as shown. Tumour volumes were monitored over time after treatment. Mean tumour volume (+SD) was determined for each treatment group ($n = 8$ each group). Note that the mean tumour volumes in the Ad/Bcl-XL shRNA-treated group differed significantly from those in the 2 control-treated groups ($*p < 0.05$); (*b*) Serum transaminase levels (mean ± SD) 2 days after last treatment. The levels were similar in all 3 treatment groups. (*c*) Histopathologic appearance of organs (heart, kidney, liver and lung) and tumour samples collected from euthanized mice. No obvious histopathologic damage was seen in any organ tissues, and no obvious inflammation was seen in any tumour samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

regional as opposed to systemic administration of adenovirus reduced inflammation, increased gene transfer efficiency and enhanced the potential for therapeutic effects[21]; however, another study demonstrated the possibility that the rapid, postinjection induction of proinflammatory genes might still initiate an inflammatory reaction and produce uncertain effects on tumour growth *in vivo*.[21,22] With this in mind, we looked for obvious signs of inflammatory reaction in adenovirally treated mouse tumours in the present study and found none. The implication is that tumour growth may have been inhibited by Ad/Bcl-XL shRNA-mediated induction of apoptosis.

Another potential concern and drawback to adenoviral RNA interference therapy is the potentially incomplete transduction of tumour cells after intratumoural adenovirus administration. It is to be expected that untransduced tumour cells, and thus any residual tumour, will grow. How such residual tumours respond to repeat treatment with the same adenoviral vector will depend on their potential for developing resistance to it. If the tumour cells remain as the parental cells innoculated, then it is conceivable that the residual tumour cells would respond to repeat adenoviral treatment.[23] However, repeated adenoviral treatment has been known to put selection pressure on resistant tumour cells, resulting in acquired resistance to the adenoviral vector, the therapeutic gene, or in some cases both.[24] In that case, an alternative strategy for treating the resistant tumours would be necessary.[7]

A third possible obstacle to adenovirus-mediated RNA interference therapy is tissue or tumour specificity. The promoter that we used in our study-the RNA polymerase III promoter U6-specifically drives, the expression of small RNA and has no specificity for the RNA of cancer cells.[25–27] Therefore, selecting a target gene that is highly expressed in tumour tissues but not in normal tissues would be important for avoiding damage to normal tissues. In one recent study, RNA interference was successfully induced by a modified prostate-specific promoter.[28] Although this approach could only be applied to certain tissues, the concept of promoter-based tumour targeting may nevertheless provide a further means of improving the safety profile of Bcl-XL shRNA therapy.

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References

1. Yin XM. Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways. Cell Res 2000;10:161–7.
2. Huang DC Strasser A. BH3-only proteins-essential initiators of apoptotic cell death. Cell 2000;103:839–42.
3. Kim IK, Jung YK, Noh DY, Song YS, Choi CH, Oh BH, Masuda ES, Jung YK. Functional screening of genes suppressing TRAIL-induced apoptosis: distinct inhibitory activities of Bcl-XL and Bcl-2. Br J Cancer 2003;88:910–17.
4. Huang X, Lin T, Gu J, Zhang L, Roth JA, Stephens LC, Yu Y, Liu J, Fang B. Combined TRAIL and Bax gene therapy prolonged survival in mice with ovarian cancer xenograft. Gene Ther 2002;9:1379–86.
5. Gu J, Zhang L, Huang X, Lin T, Yin M, Xu K, Ji L, Roth JA, Fang B. A novel single tetracycline-regulative adenoviral vector for tumour-specific Bax gene expression and cell killing in vitro and in vivo. Oncogene 2002;21:4757–64.
6. Kagawa S, Pearson SA, Ji L, Xu K, McDonnell TJ, Swisher SG, Roth JA, Fang B. A binary adenoviral vector system for expressing high levels of the proapoptotic gene bax. Gene Ther 2000;7:75–9.
7. Zhu H, Zhang L, Huang X, Davis J, Jacob D, Teraishi F, Chiao P, Fang B. Overcoming acquired resistance to TRAIL in DLD-1 colon cancer cells by chemotherapeutic agents and NFB inhibitor via distinct mechanisms. Mol ther 2004;9:666–73.
8. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. J Biol Chem 1999;274:2225–33.
9. Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci USA 1998;95:4997–5002.
10. Maurer CA, Friess H, Buhler SS, Wahl BR, Graber H, Zimmermann A, Buchler MW. Apoptosis inhibiting factor Bcl-xL might be the crucial member of the *Bcl-2* gene family in colorectal cancer. Dig Dis Sci 1998;43:2641–8.
11. Shimizu S, Eguchi Y, Kosaka H, Kamiike W, Matsuda H, Tsujimoto Y. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. Nature 1995;374:811–13.
12. Zhu H, Guo W, Zhang L, Davis JJ, Wu S, Teraishi F, Cao X, Smythe WR, Fang B. Enhancing TRAIL-induced apoptosis by Bcl-X(L) siRNA. Cancer Biol Ther 2005;4:393–7.
13. Zhu H, Guo W, Zhang L, Davis JJ, Teraishi F, Wu S, Cao X, Daniel J, Smythe WR, Fang B. Bcl-XL small interfering RNA suppresses the proliferation of 5-fluorouracil-resistant human colon cancer cells. Mol Cancer Ther 2005;4:451–6.
14. Lu PY, Xie F, Woodle MC. In vivo application of RNA interference: from functional genomics to therapeutics. Adv Genet 2005;54:117–42.
15. Wall NR Shi Y. Small RNA: can RNA interference be exploited for therapy? Lancet 2003;362:1401–3.
16. Meterissian SH, Kontogiannea M, Al Sowaidi M, Linjawi A, Halwani F, Jamison B, Edwardes M. Bcl-2 is a useful prognostic marker in Dukes' B colon cancer. Ann Surg Oncol 2001;8:533–7.
17. Davis JJ, Wang L, Dong F, Zhang L, Guo W, Teraishi F, Xu K, Ji L, Fang B. Oncolysis and suppression of tumour growth by a GFP-expressing oncolytic adenovirus controlled by an hTERT and CMV hybrid promoter. Cancer Gene Ther 2006;13:720–3.
18. Liu YP, Dambaeva SV, Dovzhenko OV, Garthwaite MA, Golos TG. Stable plasmid-based siRNA silencing of gene expression in human embryonic stem cells. Stem Cells Dev 2005;14:487–492.
19. Niola F, Evangelisti C, Campagnolo L, Massalini S, Bue MC, Mangiola A, Masotti A, Maira G, Farace MG, Ciafre SA. A plasmid-encoded VEGF siRNA reduces glioblastoma angiogenesis and its combination with interleukin-4 blocks tumour growth in a xenograft mouse model. Cancer Biol Ther 2006;5:174–9.
20. Benihoud K, Esselin S, Descamps D, Jullienne B, Salone B, Bobe P, Bonardelle D, Connault E, Opolon P, Saggio I, Perricaudet M. Respective roles of TNF-α and IL-6 in the immune response-elicited by adenovirus-mediated gene transfer in mice. Gene Ther 2007;14:533–44.
21. Crettaz J, Berraondo P, Mauleon I, Ochoa L, Shankar V, Barajas M, van Rooijen N, Kochanek S, Qian C, Prieto J, Hernandez-Alcoceba R, Gonzalez-Aseguinolaza G. Intrahepatic injection of adenovirus reduces inflammation and increases gene transfer and therapeutic effect in mice. Hepatology 2006;44:623–32.
22. Zirger JM, Barcia C, Liu C, Puntel M, Mitchell N, Campbell I, Castro M, Lowenstein PR. Rapid upregulation of interferon-regulated and chemokine mRNAs upon injection of 108 international units, but not lower doses, of adenoviral vectors into the brain. J Virol 2006;80: 5655–9.
23. Kagawa S, He C, Gu J, Koch P, Rha SJ, Roth JA, Curley SA, Stephens LC, Fang B. Antitumour activity and bystander effects of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. Cancer Res 2001;61:3330–8.
24. Zhang L, Gu J, Lin T, Huang X, Roth JA, Fang B. Mechanisms involved in development of resistance to adenovirus-mediated proapoptotic gene therapy in DLD1 human colon cancer cell line. Gene Ther 2002;9:1262–70.
25. Hui EK, Yap EM, An DS, Chen IS, Nayak DP. Inhibition of influenza virus matrix (M1) protein expression and virus replication by U6 promoter-driven and lentivirus-mediated delivery of siRNA. J Gen Virol 2004;85:1877–84.
26. Kwak YD, Koike H, Sugaya K. RNA interference with small hairpin RNAs transcribed from a human U6 promoter-driven DNA vector. J Pharmacol Sci 2003;93:214–17.
27. Spankuch B, Matthess Y, Knecht R, Zimmer B, Kaufmann M, Strebhardt K. Cancer inhibition in nude mice after systemic application of U6 promoter-driven short hairpin RNAs against PLK1. J Natl Cancer Inst 2004;96:862–72.
28. Song J, Pang S, Lu Y, Yokoyama KK, Zheng JY, Chiu R. Gene silencing in androgen-responsive prostate cancer cells from the tissue-specific prostate-specific antigen promoter. Cancer Res 2004;64: 7661–3.