

Adenovirus-mediated down-regulation of X-linked inhibitor of apoptosis protein inhibits colon cancer

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Abstract

Our previous studies and those of others have indicated that *X-linked inhibitor of apoptosis protein (XIAP)* holds promise as a target gene in colon cancer gene therapy. In this study, we constructed an adenoviral vector to deliver small hairpin RNA (shRNA) against XIAP (XIAP-shRNA) into colon cancer cells and tested its therapeutic efficacy *in vitro* and *in vivo*. We first confirmed an overexpression of XIAP in colon cancer cells and human cancer tissues. We then designed XIAP-small interfering RNA (siRNA) and confirmed the knockdown effect of these siRNAs in colon cancer cells. The sequences of the effective siRNAs were converted into shRNA and then packed into replication-deficient adenoviral vectors using BLOCK-iT Adenoviral RNAi Expression System to generate Adv-XIAP-shRNA. Infection of HT29 and HCT116 cells with Adv-XIAP-shRNA led to enhanced caspase-3 activity, which was associated with increased apoptosis and reduced cell proliferation. The therapeutic effect of Adv-XIAP-shRNA was then tested in xenograft tumors in nude mice. We showed that treatment of the xenograft tumors derived from HCT116 cells with Adv-XIAP-shRNA resulted in a retardation of tumor growth, which was associated with enhanced apoptosis, increased caspase-3 activity, and reduced expression of proliferating cell nuclear antigen in the tumor tissues. Treatment of xenograft tumors with Adv-XIAP-shRNA did not affect the

expressions of inflammatory cytokines in tumor-bearing mice. Thus, Adv-XIAP-shRNA-mediated down-regulation of XIAP exerts a therapeutic effect in colon cancer by promoting apoptosis and inhibiting proliferation of colon cancer cells, and the antitumor effect of Adv-XIAP-shRNA was unlikely to be related to virus-induced immune response. [Mol Cancer Ther 2009;8(9):2762–70]

Introduction

Current therapeutic options for advanced colorectal cancer are suboptimal; thus, more efficient treatment approaches are needed. Poor response to current cancer treatment modalities is attributed partially to resistance of cancer cells to therapy-induced apoptosis; thus, dysregulated apoptosis has been mechanistically linked to the development and treatment failure of many solid tumors. Apoptosis is normally regulated by a complex network of signaling pathways. Inhibitor of apoptosis (IAP) family represents a group of potent apoptosis regulators, among which X-linked IAP protein (XIAP) is the most potent endogenous IAP. We have conducted a series of studies showing that genetic down-regulation of XIAP in colon cancer cells increases the sensitivity of the cancer cells to peroxisome proliferator-activated receptor γ ligand-induced apoptosis and growth inhibition (1–4). Studies by others have also shown that down-regulation of XIAP leads to a sensitization of cancer cells to apoptosis, reduced angiogenesis, and tumorigenicity (5–8). Thus, XIAP can be a potential target for colon cancer gene therapy.

From therapeutic perspective, small interfering RNA (siRNA)-mediated RNA interference (RNAi) is a powerful technique that can significantly down-regulate oncogenes; thus, it constitutes a promising approach for cancer gene therapy (9, 10).

Adenoviral vectors can efficiently transduce gene into a broad range of cell types and have been used extensively as gene delivery vehicles. Adenoviral vectors carrying small hairpin RNA (shRNA) expression cassettes have been developed and tested in several studies. Adv-shRNA was found to achieve high delivery efficacy and long-term inhibition of XIAP *in vitro* or *in vivo* in several cancers, such as hepatocellular carcinoma, glioma, and breast cancer (11–14).

In this study, we develop a recombinant adenoviral vector to express a U6 promoter-driven shRNA that targets XIAP (Adv-XIAP-shRNA) and tested its efficacy *in vitro* and xenograft tumors in nude mice.

Materials and Methods

Chemicals, Reagents, and Cell Lines

All human colon cancer cell lines were purchased from the American Type Culture Collection. Human embryonal kidney 293A cell line, Trizol, PCR-related reagents, and all

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cell culture-related materials were purchased from Invitrogen. WST-1 reagent (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4]-2H-tetrazolium, monosodium salt) was purchased from Roche Diagnostics. Annexin V staining kit was from BD Biosciences. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-XIAP were purchased from Abcam, Inc. Other primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Caspase-3 Assay kit and all chemical reagents were purchased from Sigma unless otherwise stated.

Detection of XIAP in Human Colon Cancer Tissues by Immunohistochemistry

Sixteen pairs of human colon cancer tissues and corresponding noncancerous colonic tissues were used to detect the expression of XIAP by immunohistochemistry. These tissues were obtained from eight males and eight females, ages between 48 and 74 y (mean age, 60 y). Colon adenocarcinomas were classified as well ($n = 4$), moderately ($n = 9$), and poorly ($n = 3$) differentiated according to standard criteria. Tissues were fixed in 10% neutral formalin, embedded in paraffin, and cut into 4- μ m sections. Immunostaining for XIAP was done as described in our previously published article (2).

Written consents were obtained before tissue collection, and the study was approved by the Human Ethics Committees of the University of Hong Kong.

Cell Culture and Adenoviral Infection *In vitro*

HCT116 and HT29 were grown to 70% to 80% confluence before being infected with adenoviral vectors. Cells were initially incubated with adenoviral vectors for 4 h in serum-free medium followed by incubation with complete growth medium for indicated time.

Constriction of Recombinant Adenovirus Vectors and Amplification of Viral Vectors

Replication-incompetent recombinant adenoviruses expressing shRNAs were constructed using BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's instructions. The adenoviral vectors produced with this system are based on the second-generation vectors (15). Expression of the shRNA is controlled by the human U6 promoter (16, 17).

To design shRNA against XIAP, we first designed four pairs of siRNA based on the full-length XIAP (accession number: NM_001167) using the web-based siRNA designer.⁵ The oligonucleotides were synthesized by Prologo (Sigma-Aldrich). After confirming the knockdown efficacy of these designed siRNA-XIAP sequences by Western blot, the sequences that could effectively knock down XIAP were converted to shRNA sequences by adding hairpin sequences.

One ssDNA oligonucleotide with an additional sequence CACC at 5'-end (5'-CACCGAAGCATCATACTATAACTGACGAATCAGTTATAGTATGATGCTTC-3'), the other encoding complementary sequence, and an additional sequence AAAA at 5'-end (5'-AAAAGAAGCATCATACTATAACTGATTCGTCAGTTATAGTATGATGCTTC-3') were annealed and then the generated dsDNA oligonucleotide was cloned

into the pENTR/U6 vector using BLOCK-iT U6 RNAi Entry Vector kit. The U6 RNAi cassette in the pENTR/U6 XIAP-shRNA vector was transferred to the adenoviral expression plasmid by LR recombination reaction using Gateway LR Clonase II Enzyme Mix and pAd/BLOCK-iT-DEST Gateway Vector kit. The isolated adenoviral expression clones were then digested with *PacI* to expose the inverted terminal repeats and transfected into 293A cells using Lipofectamine 2000 (Invitrogen) to produce crude adenoviral stocks.

Large-scale amplification of adenoviral vectors was conducted in 293A cells as we described previously (18). The titer of the purified virus was determined by standard plaque-forming assay on 293A cells as described (19, 20). The viral titer was expressed as plaque-forming units (pfu)/mL. An adenovirus expressing β -galactosidase was used as a control (Adv-LacZ), which was used to test the transduction efficiency by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) staining assay as described (18).

Cell Cycle Analysis, Proliferation Assay, and Apoptosis Detection

Cell cycle was detected by propidium iodide (PI) staining and fluorescence-activated cell sorting analysis, as described (21), and the *in vitro* cell proliferation was done by WST-1 assay as we previously reported (3).

Apoptosis was quantified by staining cells with Annexin V-FITC/PI, according to the manufacturer's protocol, and measured by flow cytometry. Cells undergoing apoptosis showed an increase in Annexin V binding. Briefly, cells were seeded at 3×10^5 per well in a six-well plate and transduced with Adv-XIAP-shRNA or Adv-LacZ-shRNA for 48 h. Cells were trypsinized, washed with PBS, resuspended in Annexin V-FITC and PI staining solution, and incubated for 15 min at room temperature in the dark. Samples were immediately analyzed on a FACSCalibur cytometer. A minimum of 10,000 events per sample was acquired and subsequently analyzed with CellQuest software.

Caspase-3 Activity Assay

Caspase-3 activity in the cell lysates and tissue homogenates was assessed according to the kit instruction and as we described (18), and the results were expressed as nmol pNA released per hour per mg of protein (nmol pNA/h/mg).

Determination of Protein Expression by Western Blotting

For detection of XIAP in human colon tissues, total tissue lysates were extracted from seven pairs of surgically excised human colon cancer tissues and corresponding noncancerous colonic tissues, as described (2, 3).

For detection of XIAP in human colon cancer cell lines, total cell lysates were extracted from cultured colon cancer cells treated with or without Adv-XIAP-shRNA using radioimmunoprecipitation assay buffer (Sigma) supplemented with 1% of protease inhibitor cocktail, as reported (3). Standard Western blot analysis was done to detect the expression of target proteins using GAPDH as a loading control.

Xenograft Tumorigenesis in Nude Mice

Use of animals in this study was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

⁵ <http://www.dharmacon.com/sidesign>

To establish xenograft tumors, 1×10^7 HCT116 cells were injected s.c. into the dorsal flank of 6-wk-old male athymic nude mice (BALB/*c nu/nu*). When the tumors reached ~ 5 mm in diameter, each mouse was then given intratumoral injection of Adv-XIAP-shRNA or Adv-LacZ at a dose of 5×10^8 pfu/mouse for three times in a week, with the accumulative dose of 1.5×10^9 pfu/mouse. Tumor sizes were measured every other day, and tumor volume (mm^3) was calculated by the following formula: volume = (shortest diameter)² \times longest diameter / 2. On the day of harvest, the tumor tissues were removed, size was measured, and weight was determined.

Harvested tumor tissues were either snap frozen for protein and RNA analysis or fixed in 10% neutral formalin for histology and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay as reported previously (3).

Measurement of Inflammatory Cytokines in Mouse Serum by ELISA

Whole blood was drawn from the tumor-bearing mice at the time of sacrifice and immediately centrifuged at $800 \times g$ at 4°C for 5 min. The supernatant was collected and stored at -80°C and subsequently analyzed. Serum concentrations of tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β were measured by ELISA-based assays using commercial kits (R&D Systems) according to the manufacturer's instructions.

Measurement of Inflammatory Cytokines in Xenograft Tumor Tissues Using a Quantitative Real-time PCR

Harvested xenograft tumor tissues were used to extract total RNA using Trizol reagent, and real-time PCR was done as we previously described (3, 4). The primer sequences for *IL-1 β* , *TNF- α* , *IL-4*, *IL-10*, and *IL-13* used for PCR were listed in Table 1. The results were standardized to GAPDH.

Statistical Analysis

Data were expressed as mean \pm SD. All statistical analyses were conducted using the Statistical Package for the Social Sciences 16.0 package for Windows. Student's independent sample *t* test and one-way ANOVA with Dunnett's *t* test were used for comparisons. A *P* value of <0.05 was considered statistically significant.

Results

Expression of XIAP in Colon Cancer Cells and Tissues

As shown in Fig. 1A, an ample amount of XIAP was detected in six human colon cancer cell lines. We examined the expression of XIAP in 16 pairs of human colon cancer and corresponding noncancerous colonic mucosa by Western blot analysis and observed that the majority of the tumor tissues ($\sim 80\%$) exhibited increased expression of XIAP relative to their corresponding noncancerous controls. The representative Western blots were shown in Fig. 1B. By immunohistochemistry, richer expression of XIAP was found in moderately and poorly differentiated colon cancer tissues (Fig. 1C, *c* and *d*, respectively) compared with noncancerous colonic mucosa (Fig. 1C, *b*). As a positive control, strong XIAP expression was noted in human spleen tissue (Fig. 1C, *a*).

These results confirm that XIAP is overexpressed in human colon cancer. We thus attempted to down-regulate XIAP in colon cancer cells and to investigate whether such an approach would offer any antiproliferative effect against colon cancer. To achieve this goal, we designed shRNA and packed it into an adenoviral vector to generate Adv-XIAP-shRNA. By using the following experiments, we tested the efficacy of this viral vector in colon cancer cells and in a xenograft tumor model in nude mice.

Verification of the Efficiency of Adv-XIAP-shRNA on Silencing Endogenous XIAP

The knockdown efficiency of the designed four pairs of siRNA was tested in HT29 and HCT116 cells. As shown in Fig. 2A, all four pairs of siRNA-XIAP efficiently knocked down XIAP in both cell lines. The sequences of these siRNAs were converted to shRNA sequences by adding hairpin sequences. The shRNA sequences were then packed into pENTR/U6 vector and used for viral vector construction, as described in Materials and Methods.

To test the infection efficiency of the adenoviral vector, HT29 and HCT116 cells were infected with various concentrations of Adv-LacZ, ranging from 10 multiplicities of infection (MOI) to 500 MOI. After 48 hours, infected cells were stained with X-Gal staining kit, and the β -galactosidase-positive cells were calculated. Infection of HT29 and HCT116 cells with 100 MOI of Adv-LacZ produced maximal infection

Table 1. Primer list

Gene name	Genbank accession no.	Sequences
<i>TNF-α</i>	NM_000594	Forward: ATGAGCACTGAAAGCATGATCC Reverse: GAGGGCTGATTAGAGAGAGGTC
<i>IL-1β</i>	NM_000576	Forward: CTCGCCAGTGAAATGATGGCT Reverse: GTCGGAGATTCGTAGCTGGAT
<i>IL-4</i>	NM_000589	Forward: CCAACTGCTTCCCCCTCTG Reverse: TCTGTTACGGTCAACTCGGTG
<i>IL-10</i>	NM_000572	Forward: ACTTTAAGGGTTACCTGGGTTGC Reverse: TCACATGCCCTTGATGTCTG
<i>IL-13</i>	NM_002188	Forward: GAAGGCTCCGCTCTGCAAT Reverse: TCTGGGCTCTCTCGATGGCA

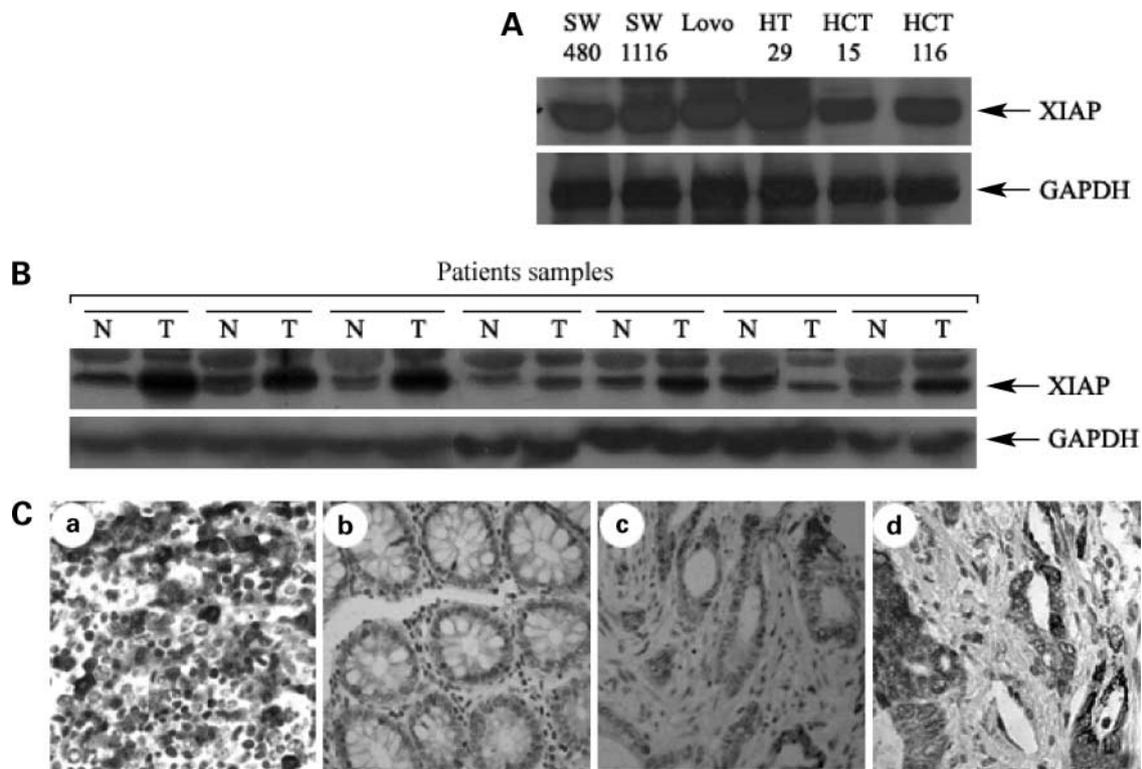


Figure 1. Expression of *XIAP* in human colon cancer cell lines and colon tissues. **A**, expression of *XIAP* in six human colon cancer cell lines by Western blot. **B**, expression of *XIAP* in seven pairs of human colon cancer tissues (*T*) and matched noncancerous colonic tissues (*N*). **C**, expression of *XIAP* was also examined by immunohistochemistry in human normal colonic mucosa (*b*) and moderately (*c*) and poorly (*d*) differentiated colon cancer tissues. *a*, human spleen was used as a positive control. Representative photos are shown. Original magnification, $\times 200$.

rate (63.5% and 77.6%, respectively; Fig. 2B) and minimal cytotoxicity. These data indicated that the adenoviral vector system is capable of efficiently transferring target gene into human colon cancer cells. We therefore chose 100 MOI of the viral vectors for subsequent studies unless otherwise stated.

We then tested the inhibitory efficiency of Adv-XIAP-shRNA on the expression of *XIAP* in HT29 and HCT116 cells. As shown in Fig. 2C, infection of cells with Adv-XIAP-shRNA for 48 hours significantly suppressed the endogenous expression of *XIAP*, showing a strong silencing ability of the Adv-XIAP-shRNA. The viral construct was also effective in down-regulating endogenous *XIAP* in other colon cancer cell lines (data not shown).

We also showed that Adv-XIAP-shRNA was not able to alter the expression of other IAP members, including *cIAP2*, *cIAP1*, and *survivin* (data not shown).

Effect of XIAP Silencing on Cell Proliferation and Apoptosis

We then did studies to investigate whether down-regulation of *XIAP* expression would affect the survival of colon cancer cells. HCT116 cells were infected with Adv-XIAP-shRNA or Adv-LacZ for 48 hours, and cell proliferation was determined by WST-1 assay. Infection with Adv-XIAP-shRNA produced a dose-dependent inhibition of cell growth (data not shown).

With 100 MOI of Adv-XIAP-shRNA, cell viability was reduced by 26.5% (Fig. 3A).

Together with reduced proliferation, there was an increased apoptosis in Adv-XIAP-shRNA-treated cells, as shown by Annexin V assay (Fig. 3B). Associated with increased apoptosis was an increased caspase-3 activity: treatment of cells with 100 MOI of Adv-XIAP-shRNA led to a 66.5% increase in caspase-3 activity (Fig. 3D).

By flow FACScan, no changes were found in cell cycle distribution following Adv-XIAP-shRNA treatment but a dramatic increase in the sub- G_0 - G_1 population was observed, also indicating apoptosis (Fig. 3C).

Adv-XIAP-shRNA Suppressed the Growth of Xenograft Tumors in Nude Mice

To explore the effects of Adv-XIAP-shRNA *in vivo*, colon cancer xenografts were generated by injecting HCT116 cells s.c. into the nude mice. The xenograft tumors were treated with Adv-XIAP-shRNA. Compared with Adv-LacZ-treated tumors, Adv-XIAP-shRNA caused a marked inhibition of tumor growth as shown by a reduced tumor weight and volume (Fig. 4). The average weight of the harvested xenograft tumors from Adv-XIAP-shRNA group was lighter than tumors from Adv-LacZ group (0.41 g versus 0.71 g, respectively; $P < 0.001$; Fig. 4A), and the average volume of the Adv-XIAP-shRNA-treated tumors was smaller than that

of the Adv-LacZ-treated tumors ($493.26 \pm 128.53 \text{ mm}^3$ versus $851.89 \pm 217.41 \text{ mm}^3$, respectively; $P < 0.01$; Fig. 4B). The overall growth speed of the Adv-XIAP-shRNA-treated xenograft tumors was much slower compared with that of the Adv-LacZ-treated tumors (Fig. 4C). No other histologic abnormalities and changes in body weights were found (data not shown).

Adv-XIAP-shRNA Induced Apoptosis and Suppressed the Proliferation of Xenograft Tumors

To investigate the mechanisms of tumor suppression by Adv-XIAP-shRNA, we measured apoptosis, cell proliferation index, and caspase-3 activity in the harvested tumor tissues.

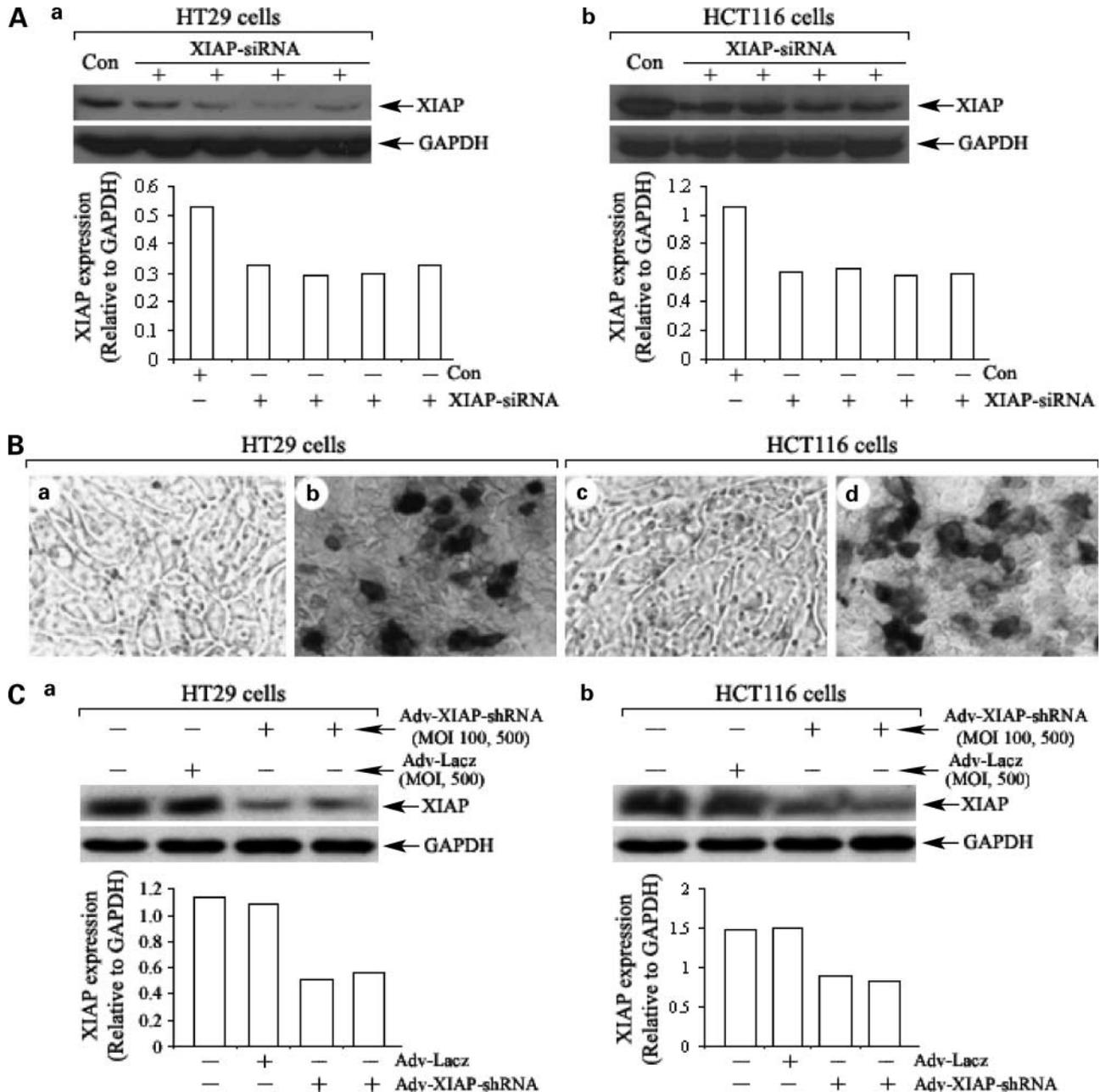


Figure 2. Verification of XIAP down-regulation in human colon cancer cells. **A**, top, knockdown of XIAP in HT29 (a) and HCT116 (b) cells by direct transfection with XIAP-siRNA. Cells without any transfection were controls (Con). Bottom, quantitative data. **B**, infection efficiency of adenoviral vector by X-Gal staining. HT29 and HCT116 cells were grown to 70% to 80% confluence and infected with (b and d) or without (a and c) 100 MOI of Adv-LacZ for 48 h followed by X-Gal staining. Blue-stained cells indicate presence of β -galactosidase gene. Results are representative of at least three experiments. **C**, efficiency of Adv-XIAP-shRNA on endogenous expression of XIAP in HT29 (a) and HCT116 (b) cells. Cells were infected with Adv-XIAP-shRNA (100 and 500 MOI) or Adv-LacZ (500 MOI) for 48 h. Cells without any infection were baseline controls. XIAP was detected by Western blot analysis. Bottom, quantitative data.

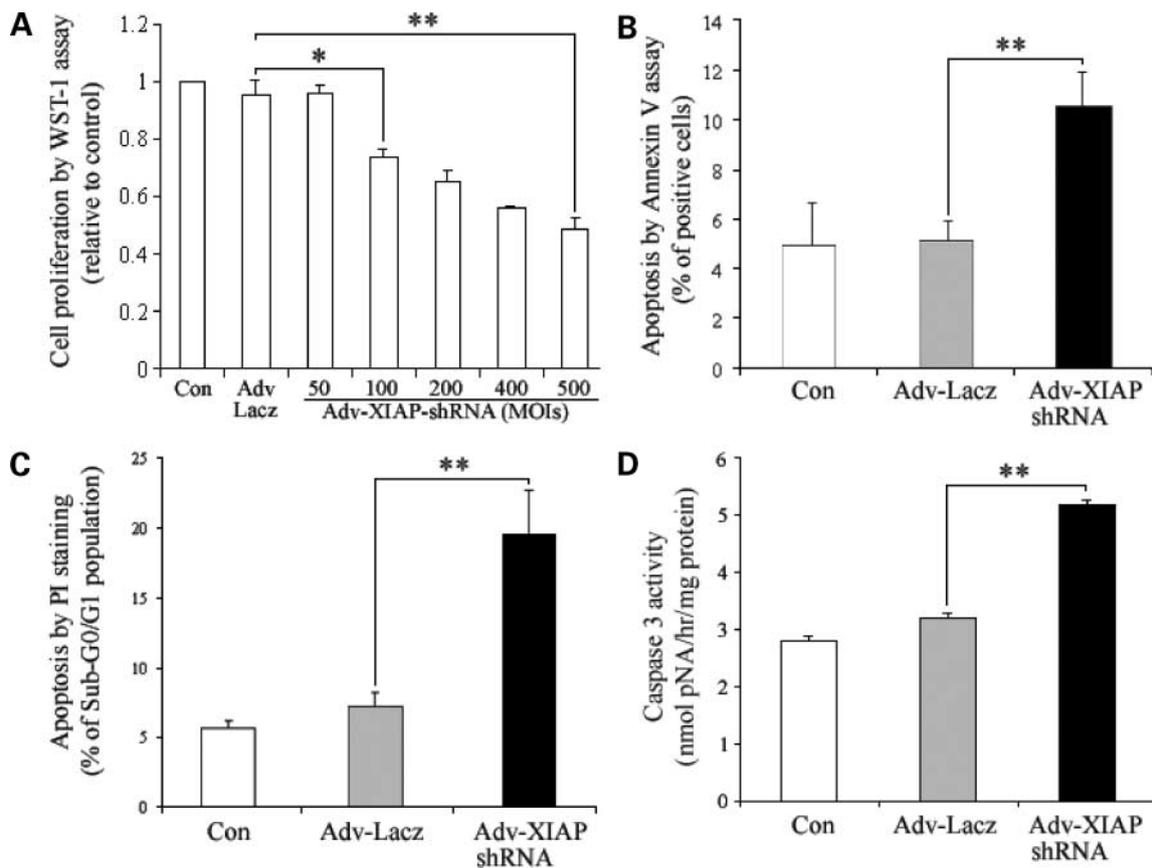


Figure 3. Effect of XIAP down-regulation on cell proliferation and apoptosis. HCT116 cells were infected with control (*Con*), Adv-Lacz (500 MOI), or various concentrations of Adv-XIAP-shRNA for 48 h. **A**, cell proliferation was determined by WST-1 assay. In apoptosis and caspase-3 activity assays, Adv-XIAP-shRNA was used at 100 MOI. Apoptosis was determined by Annexin V (**B**) and PI staining (**C**). **D**, caspase-3 activity was determined by a colorimetric assay. All experiments were repeated three times. *, $P < 0.01$; **, $P < 0.001$.

Firstly, Adv-XIAP-shRNA suppressed the expression of XIAP in tumor tissues (Fig. 5B).

Meanwhile, Adv-XIAP-shRNA induced significant apoptosis as judged by TUNEL assay (Fig. 5A, *a-c*). The increased apoptosis was associated with increased caspase-3 activity (Fig. 5A, *d*).

Together with these changes is a reduced cell proliferation index proliferating cell nuclear antigen expression in Adv-XIAP-shRNA-treated tumors (Fig. 5B).

Adv-XIAP-shRNA Did Not Affect the Inflammatory Cytokines

To determine whether the adenoviral vectors were able to induce inflammatory response in the tumor-bearing mice, we first determined the levels of *TNF- α* , *IL-1 β* , *IL-4*, *IL-10*, and *IL-13* levels in xenograft tumor tissues and found that they were not different between control, Adv-Lacz, and Adv-XIAP-shRNA groups (data not shown). We also determined the serum levels of *TNF- α* and *IL-1 β* from these mice and found no differences between three groups (data not shown).

Discussion

Targeting apoptosis including the IAP family members can be a promising approach in cancer therapy (22). Our previ-

ous and current studies have shown that XIAP is expressed at a higher level in colon cancer tissues compared with normal colonic mucosa, making XIAP a potentially useful molecular target. Indeed, targeting XIAP has been attempted in many solid tumors, including liver, breast, lung, prostate, ovarian, and colon cancers (23–29). Using an XIAP knockout cell system (30), we have previously shown that genetic down-regulation of XIAP significantly sensitized colon cancer cells to peroxisome proliferator-activated receptor γ ligand-induced cell killing both *in vitro* and *in vivo* (1–3). Thus, XIAP can be a promising target gene for colon cancer gene therapy. In the present study, we have explored the therapeutic efficacy of Adv-XIAP-shRNA in the treatment of human colon cancer.

In recent years, gene therapy is focused on several issues: improvement of gene delivery capacity, enhancement of gene targeting specificity, sustainable gene expression, and improved safety. shRNA-based gene knockdown was reported to be a useful gene silencing approach in cancer cells with a silencing efficiency ranging from 40% to 89% knockdown (31, 32). As plasmid-based gene delivery has limited delivery capacity especially *in vivo*, we generated an Adv-XIAP-shRNA and tested the therapeutic efficacy of this viral construct in colon cancer cells *in vitro* and *in vivo*. We showed that this viral construct could achieve ~70% transduction rate and

significantly suppressed the endogenous expression of XIAP *in vitro*. Further studies have shown that Adv-XIAP-shRNA infection was able to induce apoptosis and inhibit proliferation of several colon cancer cell lines. *In vivo* studies showed that this construct led to a significant retardation of the xenograft tumor growth and caused a marked down-regulation of XIAP in the tumor tissues, suggesting that direct down-regulation of XIAP by Adv-XIAP-shRNA has a potential therapeutic effect in colon cancer. These results are largely consistent with previous reports in lung, prostate, and ovarian cancer cells (25–27).

Caspase-3 is the key downstream component of different major apoptotic pathways. Our data revealed that Adv-XIAP-shRNA induced a significant activation of caspase-3 *in vitro* as well as *in vivo*, and the caspase-3 activation was associated with enhanced apoptosis and tumor growth retardation. Overexpression of XIAP was associated with blockade of caspase-3 activity in colon cancer cells (33). Our published data derived from a microarray analysis indicated that down-regulation of XIAP in colon cancer cells was associated with a down-regulation of several cell proliferation-related genes and an up-regulation of apoptosis-associated genes (4). One aspect that warrants further investigation is the discrepancy between the nonimpressive *in vitro* growth inhibition by 100 MOI of Adv-XIAP-shRNA, which reduced cell viability by 26.5%, and the impressive *in vivo* tumor growth suppres-

sion. A better *in vivo* therapeutic response may be partially attributable to the higher accumulative dose of Adv-XIAP-shRNA (1.5×10^9 pfu/mouse). More studies using different cell lines may reveal new information necessary to understand this question.

Nevertheless, our data indicated that reduced cellular proliferation and enhanced apoptosis mediated by activation of caspase-3 were likely to be mechanistically responsible for the observed antitumor effect of Adv-XIAP-shRNA. In addition, these *in vitro* and *in vivo* studies have further emphasized an essential role of XIAP in maintaining cell survival ability in colon cancer, thus forming a strong basis for further exploring the therapeutic benefit of XIAP targeting.

As gene therapy using adenoviral vectors may be limited by their proinflammatory responses and adverse side effects *in vivo*, we paid particular attention to the side effect of adenoviral vectors in tumor-bearing mice but did not observe any serious adverse reaction, such as liver toxicity and unexpected animal death. We examined the expressions of several inflammatory cytokines both in xenograft tumor tissues and bloodstream and found that Adv-XIAP-shRNA did not induce appreciable changes in these cytokines, suggesting that Adv-XIAP-shRNA-mediated antitumor effect was independent of immune response. However, as this study only lasted for 4 weeks, the possible development of immune

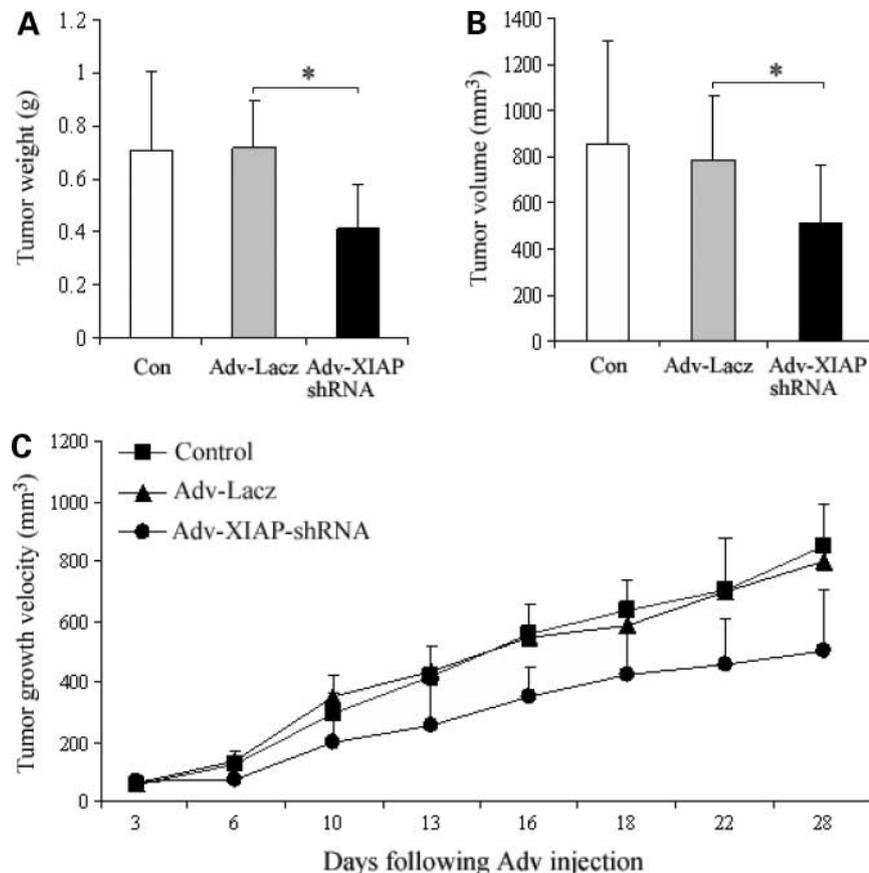


Figure 4. Effects of Adv-XIAP-shRNA on the growth of xenograft colon cancers in nude mice. Eighteen nude mice were s.c. injected with HCT116 cells. When the xenograft tumor grew to 5 mm in diameter, a total of 1.5×10^9 pfu of Adv-XIAP-shRNA or Adv-Lacz were injected intratumorally to each mouse. Mice receiving PBS injection were controls (Con). Tumor volumes were monitored and tumor tissues were harvested 28 d after the initial viral vector injection. **A**, average weight. **B**, average volume of the harvested tumors ($n = 6$, for each group). *, $P < 0.01$. **C**, growth curve of the xenograft tumors.

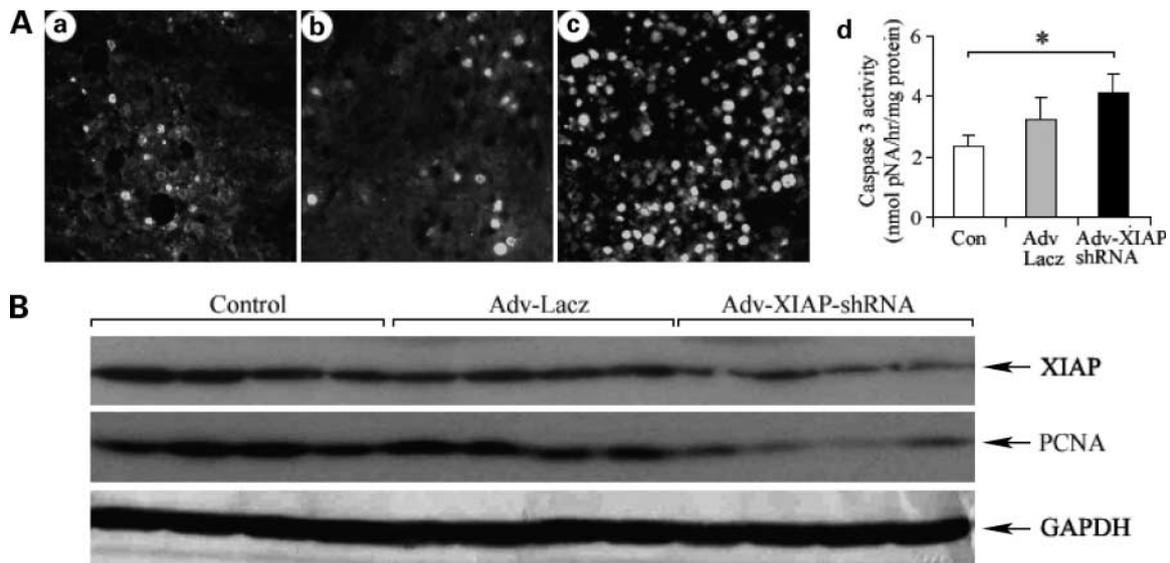


Figure 5. **A**, apoptosis and caspase-3 activity in xenograft tumor tissues. Apoptosis was detected by TUNEL assay in xenograft tumors treated with PBS (a), Adv-LacZ (b), and Adv-XIAP-shRNA (c). Original magnification, $\times 100$. **d**, caspase-3 activity in the tissue lysates was measured using a colorimetric assay. *, $P < 0.01$. **B**, expression of XIAP and proliferating cell nuclear antigen (PCNA) in the xenograft tumor tissues by Western blot. Each lane represents a separate tumor tissue.

response against the adenoviral vectors following long-term injection could not be excluded.

In addition, we did not note any down-regulating effect of Adv-XIAP-shRNA on other members of IAP family, such as cIAP1, cIAP2, and survivin, a nonspecific drawback observed in antisense-based XIAP targeting (13). These observations suggested that adenoviral-mediated delivery of XIAP is a specific and efficient gene delivery approach and may hold a promising potential in colon cancer gene therapy. However, we must note that intratumoral injection of the adenoviral vectors is practically impossible in real clinical setting, and leakage of the virus into neighboring tissues and/or bloodstream may be inevitable. Thus, a more efficient gene delivery approach has to be developed in future gene therapy.

In conclusion, we have shown that XIAP plays an important role in the regulation of apoptosis and proliferation of colon cancer cells and may thus be a promising molecular target for colon cancer gene therapy. Adenoviral vector-based delivery of XIAP is a safe and efficient therapeutic approach against human colon cancer. Further large-scale studies in more cancer systems will be necessary to unveil the molecular mechanisms of XIAP targeting in cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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