

Targeting X-linked inhibitor of apoptosis protein inhibits pancreatic cancer cell growth through p-Akt depletion

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Abstract

AIM: To determine whether lentivirus-mediated shRNA targeting the X-linked inhibitor of apoptosis protein (XIAP) gene could be exploited in the treatment of pancreatic cancer.

METHODS: Human pancreatic cancer cells Panc-1, Mia-paca2, Bxpc-3 and SW1990, infected with lentivirus, were analyzed by real-time polymerase chain reaction (PCR). Western blotting was used to examine XIAP protein levels, survivin and p-Akt to confirm the result of real-time PCR and determine the possible

mechanism. The 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure IC₅₀ to determine chemosensitivity to the chemotherapeutic drugs 5-fluorouracil (5-FU) and gemcitabine. A colony assay, MTT assay and a tumorigenicity experiment were used to study cell proliferation *in vitro* and *in vivo*. Caspase-3/7 activity, 4',6-diamidino-2-phenylindole-staining and flow cytometric measurements were used to study apoptosis in SW1990 cells.

RESULTS: XIAP proteins were found to be differentially expressed among pancreatic cancer cell lines Panc-1, Mia-paca2, Bxpc-3 and SW1990. Data of real-time PCR and Western blotting showed that XIAP was reduced persistently and markedly by lentivirus-mediated shRNA. Downregulation of XIAP by transfection with XIAP shRNA resulted in decreased p-Akt expression. XIAP shRNA also inhibited the growth of pancreatic cancer cells *in vitro* and *in vivo*, enhanced drug-induced apoptosis and increased chemosensitivity to 5-FU and gemcitabine. Results also suggest that inhibition of XIAP and subsequent p-Akt depletion may have an anti-tumor effect through attenuating the ability of cancer cells to survive.

CONCLUSION: Lentivirus-mediated gene therapy is an attractive strategy in the treatment of pancreatic cancer and justifies the use of lentivirus in pancreatic cancer gene therapy studies.

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Key words: Pancreatic cancer; Lentivirus-mediated shRNA; X-linked inhibitor of apoptosis protein; p-Akt; Gene therapy; Proliferation; Apoptosis

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INTRODUCTION

Pancreatic cancer is one of the most aggressive human malignancies, with an extremely poor prognosis and a 5-year survival rate of only approximately 5%^[1], partially due to the very little possibility of surgical resection and resistance to chemo-radiotherapy. Disordered apoptosis and abnormal proliferation have been linked with development of malignancy and treatment resistance^[2,3]. Apoptosis, also termed programmed cell death, occurs *via* extrinsic or intrinsic signal transduction pathways^[4,5]. Therefore, further understanding of the molecular mechanisms, the relationship between pancreatic cancer chemoresistance and disordered apoptosis and abnormal proliferation, can be important in trying to circumvent resistance to cancer therapy^[6].

To date, 8 human inhibitor of apoptosis protein (IAP) family members [X-linked IAP (XIAP), cIAP1, cIAP2, IAP-like protein 2, melanoma IAP, neuronal apoptosis inhibitory protein, survivin and baculovirus IAP repeats repeat-containing ubiquitin conjugating enzyme] have been identified. XIAP, a member of the IAP family, plays an important role in regulating both apoptosis and cell proliferation. XIAP is one of the most important members of the IAP family. It is highly expressed in malignant tumor cells and promotes tumor cell invasion, metastasis, growth, survival and chemoresistance. It is reported that XIAP antagonists such as second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI increase caspase activity, and not only directly induce apoptosis of many types of tumor cell lines *in vitro*, but also suppress growth of established tumors in xenograft models in mice *in vivo*, while displaying little toxicity to normal tissues. These findings validate XIAP as a target for cancer gene therapy. XIAP is a key factor in malignancy development and treatment resistance, which is associated with disordered cell apoptosis and abnormal proliferation. It is clear that XIAP is one of the most efficient caspase inhibitors of the 8 proteins, and inhibition of apoptosis by XIAP is mainly coordinated through binding to initiator caspase-9 and effector caspase-3 and caspase-7^[7-9]. In addition to caspase inhibition, XIAP induces nuclear factor- κ B and mitogen-activated protein kinase activation during transforming growth factor- β and bone morphogenetic protein receptor signaling and overexpression. Akt (protein kinase B) represents a subfamily of serine/threonine kinases that promotes cell survival^[10]. XIAP and Akt are functionally linked in maintaining homeostasis between cell death and cell proliferation^[11]. XIAP may be a core link between the cell apoptosis signaling pathway and the cell survival

signaling pathway. Targeting XIAP might simultaneously influence cell apoptosis and proliferation.

RNA interference (RNAi) is a sequence specific post-transcriptional gene silencing factor, which has been extensively used in the study of gene function and gene therapy for cancer^[12]. The use of chemically synthesized small interfering RNA (siRNA) or siRNA-encoding plasmids to produce RNAi in mammalian cells by transfection is still limited in clinical application due to some disadvantages including transient expression, and low transfection efficiency especially in non-dividing cells and when it is necessary to generate long-time gene silencing *in vivo*. A lentivirus-based vector is considered to be a promising gene delivery tool because of its ability for specific, highly stable and functional knockout of gene expression in both dividing and non-dividing cells compared with retroviral vectors. In addition, there is minimal immunogenicity associated with lentivirus vectors compared with adenoviral vectors^[13-15].

In this study, we constructed lentivirus vectors encoding shRNA targeting the human XIAP gene to study the possible mechanisms of the XIAP gene in regulating apoptosis and proliferation in pancreatic cancer. There was an inhibitory effect of XIAP gene shRNA on the growth of SW1990 cells *in vitro* and *in vivo*, which would be useful for the development of gene therapy approaches for pancreatic cancer treatment in clinical application.

MATERIALS AND METHODS

Construction and production of lentivirus vectors

Three self-complementary hairpin DNA oligos targeting XIAP mRNA and a negative control were synthesized and cloned into a lentivirus vector. A self-inactivating lentivirus vector pGCSIL-PUR (Genechem, Shanghai, China) containing a cytomegalovirus-driven puromycin and a U6 promoter upstream of the cloning restriction sites (*Age*I and *Eco*RI) was used. Three coding regions corresponding to targeting human XIAP (GenBank Accession No: NM_001167) were selected as shRNA target sequences under the guide of the shRNA design protocol. We constructed 3 shRNA-XIAP and negative control lentivirus vectors, namely Lv-X1, Lv-X2, Lv-X3 and Lv-Xnc, respectively (Table 1). Oligonucleotides were annealed and inserted between the *Age*I and *Eco*RI restriction sites of the lentivirus vector. They were confirmed by restriction mapping and DNA sequencing. Lentivirus vector DNA and packaging vectors (pHelper1.0, pHelper2.0) were then transfected into 293T cells. Forty-eight hours later, the supernatant containing the lentivirus particles was collected, filtered through the 0.45 μ m cellulose acetate filters, and the titer of lentiviruses was determined by hole-by-dilution titer assay. The virus titers produced was approximately 10^9 TU/mL.

Cell culture and infection

SW1990, Panc-1, Mia-paca2 and Bxpc-3 human pancre-

Table 1 Sequences selected as target sequences of RNA interference against human X-linked inhibitor of apoptosis protein (NM_001167)

Name	Target sequences selected	Sequence cloned into the vector
Lv-X1	GGTGAAGGTGATAAAGTAA	f: 5'-CCGGTAGGTGAAGGTGATAAAGTAATTCAGAGATTACITTTATCACCTTCACCTA TTTTIG-3' r: 5'-AATTCAAAAAATAGGTGAAGGTGATAAAGTAATCTCTTGAATTACTTTATCACCTTCACCTA-3'
Lv-X2	CTTGAGGAGTGTCTGGTAA	f: 5'-CCGGCACTTGAGGAGTGTCTGGTAATTCAGAGATTACCAGACACTCCTCAAGTGTTTTG-3' r: 5'-AATTCAAAAAACACTTGAGGAGTGTCTGGTAATCTCTTGAATTACCAGACACTCCTCAAGTG-3'
Lv-X3	GTGGTAGTCTGTTTCAGC	f: 5'-CCGGAAGTGGTAGTCTGTTTCAGCTTCAAGAGAGCTGAAACAGGACTACCACTTTTTTTG-3' r: 5'-AATTCAAAAAAAGTGGTAGTCTGTTTCAGCTCTCTTGAAGCTGAAACAGGACTACCACTT-3'
Lv-Xnc	TTCTCCGAACGTGTCACGT	f: 5'-CCGGTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTCGGAGAAATTTTG-3' r: 5'-AATTCAAAAAATCTCCGAACGTGTCACGTTCTCTTGAACGTGACACGTTCCGAGAA-3'

atic cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin) at 37 °C in a humidified incubator containing 5% CO₂. SW1990 cells were maintained in DMEM and was plated into 6-well plates at 1 × 10⁵ cells per well. Overnight when the cells reached 30%-50% confluence, they were infected with viral particles in the presence of polybrene (5 µg/mL final concentration) and ENi.S (Genechem) for 8 h at a multiplicity of infection (MOI) of 50, and then added to fresh medium. The transfected SW1990 cells were subcultured at an appropriate density in fresh DMEM and 90% of the cells were transfected at 5 d post-transfection as indicated by the expression of green fluorescent protein (GFP) (pGCSIL-GFP empty vector was used to observe the transfection efficiency of lentivirus particles). Pooled stable transfectants were established using puromycin (Sigma-Aldrich, St Louis, MI, United States) selection. Puromycin was added into the medium to select stably transfected cells at a concentration of 1 µg/mL. Puromycin-resistant colonies were picked up 14 d after transfection and stable transfectant cells were maintained in medium containing 0.5 µg/mL puromycin.

Quantification by real-time polymerase chain reaction

Total RNA was isolated using Trizol (Invitrogen). M-MLV reverse transcriptase (Fermentas) was used to create cDNA according to the manufacturer's instructions: 1 µg RNA, Oligo dt18 as primer, 42 °C for 60 min, 70 °C for 5 min. Quantitative real-time polymerase chain reaction (RT-PCR) assays were carried out using SYBR TAQ real-time kits (TaKaRa Biotechnology, Otsu, Japan) and RT-PCR amplification equipment ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, United States). The PCR primers used to detect XIAP and β-actin were as follows: XIAP, upstream primer 5'-GACAG-TATGCAAGATGAGTCAAGTCA-3', downstream primer 5'-GCAAAGCTTCTCCTCTTGACAG-3', with a product length of 93 bp; β-actin, upstream primer 5'-ACTCTTCCAGCCTTCCTTCC-3', downstream primer 5'-GTACTTGCGCTCAGGAGGAG-3', with a product length of 232 bp. The quantitative RT-PCR parameters and analysis of results were performed as normal.

Western blotting analysis

The cell extracts were prepared with lysis buffer radio

immunoprecipitation assay containing 50 mmol/L Tris-HCl, pH 7.3, 150 mmol/L NaCl, 2% NP-40, 0.5% deoxycholate, 2 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L NaF, and 1% Protease Inhibitor Cocktail (Pierce, Rockford, IL, United States). Total protein concentration was measured using the BCA assay kit (Sigma, Inc.) with bovine serum albumin as a standard according to the manufacturer's instructions. Western blotting was performed with primer and secondary antibodies: (1) Goat antihuman XIAP polyclonal antibody (R and D Systems Inc., Minneapolis, MN, United States) 1:2000, secondary antibody, 1:10 000; (2) Rabbit antihuman survivin antibody (Novus Biologicals, Inc.) 1:1000, secondary antibody, 1:10 000; (3) Rabbit antihuman p-Akt antibody (Abcam, Inc.) 1:300, secondary antibody, 1:10 000; and (4) Mouse antihuman β-actin monoclonal antibody (Sigma, Inc.) 1:10 000, secondary antibody, 1:10 000. Densitometry was performed by Quantity One image analysis software.

3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cells were plated in 96-well plates at 5 × 10³ (to test drug sensitivity) or 1 × 10³ (for the growth curves) per well. Cell growth was examined by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after lentivirus transfection once a day for 8 d. The cells were cultured in DMEM overnight to ensure that they adhered to the wall of the plates. Various concentrations of 5-fluorouracil (5-FU) or gemcitabine were added to the medium. After incubation for 72 h, 20 µL of 5 mg/mL MTT (Sigma, Inc.) was added to the medium and cultured for another 4 h, then 150 µL of dimethyl sulfoxide (Sigma, Inc.) was added into each well and shaken for 10 min. Absorbance of each well was read using a Bio-Rad model 550-microplate reader (Bio-Rad Co., CA, United States) at a wavelength of 490 nm. Semilogarithmic curves were drawn for cell survival and the logarithm of the drug concentration by SPSS16.0. The 50% inhibitory concentration (IC₅₀) was determined according to the curves.

Colony assay

Approximately 1 × 10³ non-transfected control cells and SW1990 cells stably transfected with Lv-Xnc, Lv-X1, Lv-X2, Lv-X3 were plated in 10-cm culture dishes. After 28 d, cells were fixed with methanol and stained with 0.1% crystal violet. Colonies were counted by visual inspection.

Caspase-3/7 activity

Caspase-3/7 activity was evaluated using the Caspase-Glo 3/7 Assay (Promega, Madison, United States) according to the manufacturer's instructions. Cells (1×10^5 cells per well) were seeded in a 6-well plate incubated at 37 °C overnight, then specific concentrations of 5-FU or gemcitabine were added into the medium. The ultimate concentrations for both 5-FU and gemcitabine were 1, 10 and 0.1, 1 µg/mL. Plates were further incubated at 37 °C for 72 h and luminescence was measured at 3 s delay-time, 10 s duration using SIRIUS Luminometer V3.2 (Berthold, Inc., Germany). Caspase-3/7 activity was normalized to the number of viable cells (as determined by trypan blue staining). Caspase-3/7-fold induction was determined as the ratio between caspase-3/7 activities in treated and control cells.

4',6-diamidino-2-phenylindole staining

Cells were seeded into 24-well plates on sterile round glass coverslips at a density of 2×10^4 cells per well. The cells were incubated in the medium with various concentration of 5-FU or gemcitabine for 72 h. Cells were then washed once with phosphate-buffered saline (PBS) and fixed in PBS containing 4% paraformaldehyde and 10% sucrose at room temperature for 15 min in the dark. Cells were labeled with 4',6-diamidino-2-phenylindole (DAPI) in PBS (1 µg DAPI/mL) at room temperature for 2 min in the dark. Thereafter, cells were washed twice with PBS and once with distilled water, and mounted in glycerol (60%, 4 µL). Staining was visualized *via* fluorescence microscope. The apoptosis index (AI) of cultured SW1990 cells with different lentivirus transfection was calculated using the following formula. AI (%) = apoptotic cells/total cells \times 100%.

Flow cytometric measurements

Apoptosis was measured with an annexin V-fluorescein isothiocyanate Apoptosis Detection Kit (Beyotime institute of biotechnology, China). Cells were seeded in 6-well culture plates and divided into the following groups: non-transfected control, SW1990 cells stably transfected with Lv-Xnc, Lv-X1; SW1990 + 5-FU, Lv-Xnc + 5-FU, Lv-X1 + 5-FU; SW1990 + gemcitabine, Lv-Xnc + gemcitabine, Lv-X1 + gemcitabine. Each group contained three culture flasks. When the cells were 70%-80% confluent, cells were added with 1 µg/mL 5-FU or 0.1 µg/mL gemcitabine. After 72 h, the cells were harvested and washed in cold PBS. Annexin V and PI staining were carried out using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol. Apoptotic cells were immediately analyzed by fluorescence-activated cell sorting analysis.

Tumorigenicity experiments

To determine whether the Lv-X1 silence XIAP gene could inhibit tumor development *in vivo*, non-transfected control cells, Lv-Xnc control, Lv-X1 transfected SW1990

cells (1.5×10^7 cells in 200 µL DMEM) were injected subcutaneously into the left axilla of BALB/c nude mice (6 mice per group). Tumor growth was monitored every 4 d in 2 dimensions with a vernier caliper, and tumor size was calculated according to the formula $V = a^2b/2$, where a and b are the shortest and longest diameters, respectively.

Statistical analysis

All data are expressed as mean \pm SD. Analysis was performed using analysis of variance or the Student *t* test. The relationship between XIAP protein level and IC₅₀ was analyzed by Pearson linear correlation analysis. The criterion for significance was $P < 0.05$. All the statistical analysis was performed by SPSS16.0.

RESULTS

XIAP overexpression is associated with greater chemotherapeutic drug chemoresistance

Levels of XIAP expression were highest in Panc-1 and SW1990 cell lines with a higher degree of 5-FU and gemcitabine chemoresistance than Mia-paca2 and Bxpc-3, which expressed XIAP at relatively lower levels (Figure 1A and B).

Selection of the most effective suppression XIAP specific shRNA vector

In order to exclude an off-target silencing effect mediated by specific shRNA, we designed 3 different sequences targeting XIAP and selected the most effective Lv-shRNA in this study. Real-time RT-PCR was performed after transfection and selection with puromycin. The XIAP mRNA expression in Lv-X1, Lv-X2 and Lv-X3 transfected SW1990 cells were reduced by $62.48\% \pm 7.67\%$, $49.62\% \pm 4.7\%$ and $54.47\% \pm 2.7\%$, respectively, compared with the Lv-Xnc transfected control ($P < 0.05$). In addition, no difference was observed between the Lv-Xnc control and the SW1990 control ($P > 0.05$) (Figure 1C). Western blotting revealed that the inhibition efficiencies on XIAP protein expression by Lv-X1, Lv-X2, and Lv-X3 lentivirus were consistent with that on the targeted genes' mRNA expression. XIAP protein was knocked down in Lv-X1, Lv-X2 and Lv-X3 transfected SW1990 cells, its expression demonstrated a significant reduction in Lv-X1 ($5.98\% \pm 0.7\%$), Lv-X2 ($12.32\% \pm 0.9\%$) and Lv-X3 ($13.52\% \pm 2.2\%$) transfected SW1990 cells compared with the Lv-Xnc transfected control ($P < 0.05$). In addition, no difference was observed between the Lv-Xnc control and the SW1990 control ($P > 0.05$) (Figure 1D). According to the results of RT-PCR and Western blotting, Lv-X1 was the most effective lentivirus vector and thus we used it in the following research. To validate the specificity of RNAi targeting XIAP, we also determined the level of another IAP family protein, survivin. The results showed that survivin was not affected by any constructed lentivirus (Figure 1D).

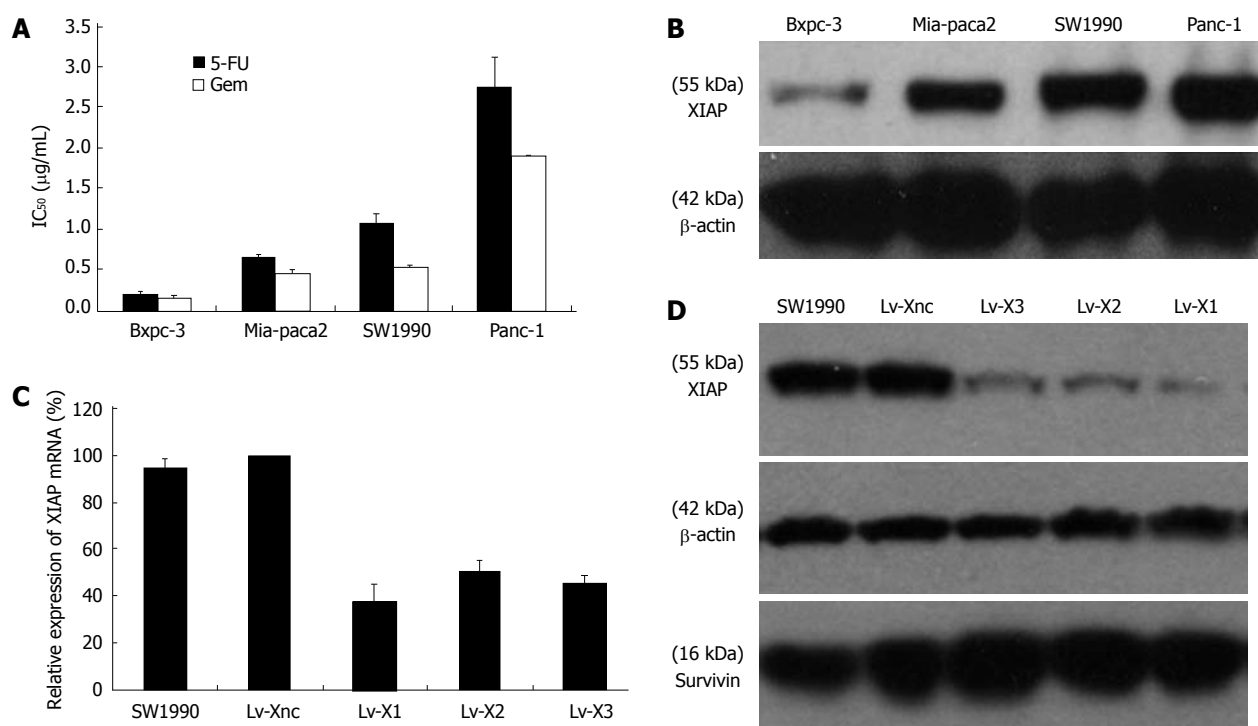


Figure 1 X-linked inhibitor of apoptosis protein expression analysis and selection of the RNAi target for X-linked inhibitor of apoptosis protein. A, B: The X-linked inhibitor of apoptosis protein (XIAP) protein level and IC₅₀ for Panc-1, SW1990, Mia-paca2 and Bxpc-3 cell lines. $R^2 = 0.87$ (Spearman correlation) between IC₅₀ and relative XIAP expression, $P < 0.05$; C: Relative expression of XIAP mRNA after transfection and selection with puromycin; D: Downregulatory effect of lentivirus-mediated RNAi on XIAP in SW1990 cells. Lv-X1, Lv-X2, Lv-X3 efficiently suppressed XIAP expression. The results also showed that survivin was not affected by any lentivirus. Lv-Xnc, which was transfected with the non-sense lentivirus vector, was set as the calibrator with the relative expression value of "1". β-actin was used as the internal loading control in three independent experiments. 5-FU: 5-fluorouracil.

Suppression of XIAP expression enhances drug-induced cytotoxicity and inhibits cell proliferation *in vitro* and *in vivo*

To determine the IC₅₀, cells were exposed to 1000, 100, 10, 1, 0.1, 0.01 and 0.001 µg/mL 5-FU or gemcitabine for 72 h. The IC₅₀ was calculated from MTT cytotoxicity assay data. Lv-X1 (0.2 ± 0.01 µg/mL for 5-FU, 0.14 ± 0.03 µg/mL for gemcitabine), Lv-X2 (0.72 ± 0.08 µg/mL for 5-FU, 0.42 ± 0.02 µg/mL for gemcitabine), Lv-X3 (0.5 ± 0.05 µg/mL for 5-FU, 0.28 ± 0.01 µg/mL for gemcitabine) inhibited the IC₅₀ significantly ($P < 0.05$ *vs* Lv-Xnc), and Lv-X1 was the most effective. Lv-Xnc control had no effect on the IC₅₀ ($P > 0.05$ *vs* SW1990 control) (Figure 2A). When compared with SW1990 and Lv-Xnc control cells, Lv-X1, Lv-X2 and Lv-X3 infected SW1990 transfected cells showed much slower growth, especially Lv-X1 infected SW1990 transfected cells. XIAP shRNA lentivirus transfection knockdown of XIAP significantly inhibited the proliferation of cultured SW1990 cells *in vitro* at the time points from day 4 to day 8 ($P < 0.05$) (Figure 2B). The result of the colony formation assay indicated that the number of colonies of Lv-X1 cells ($55.26\% \pm 3.74\%$) were much less than that of Lv-Xnc ($100.6\% \pm 5.07\%$) and non-transfected cells ($100\% \pm 6.04\%$) ($P < 0.05$) (Figure 2C). To determine whether Lv-X1 knockout of the XIAP gene could inhibit tumor development *in vivo*, SW1990 control cells, Lv-Xnc control, and Lv-X1 infected SW1990 cells were

injected into BALB/c nude mice and tumor growth was monitored every 4 d. At the time points of day 16 to day 28 after SW1990 implantation, the tumorigenicity experiments revealed that suppression of XIAP in the Lv-X1 group significantly inhibited the growth of the transplanted tumor in nude mice, which was consistent with the results achieved from the experiments *in vitro* ($P < 0.05$). For example, the tumor volume in nude mice at day 28 after the inoculation of SW1990 cells in groups of non-transfected, Lv-Xnc and Lv-X1 were 1223.28 ± 176.14 , 1173.45 ± 149.61 and 532.83 ± 84.59 mm³, respectively. When compared with SW1990 and Lv-Xnc control cells, Lv-X1 infected SW1990 transfected cells developed much smaller tumors in the nude mice ($P < 0.05$) (Figure 2D).

XIAP specific silencing enhances drug-induced activation of caspase-3/7 and enhances drug-induced apoptosis

5-FU or gemcitabine induced apoptosis in cancer cells, *via* caspase activation and inhibition of apoptosis by XIAP, was mainly coordinated through binding to effector caspase-3 and caspase-7. Thus, we sought to determine the effect of XIAP silencing on caspase activities after exposure to different concentrations of 5-FU or gemcitabine for 72 h. 5-FU or gemcitabine-induced caspase-3/7 activity was markedly increased after transfection of Lv-X1 (4.41 ± 0.51 -fold, 23.31 ± 8.14 -fold at 1,

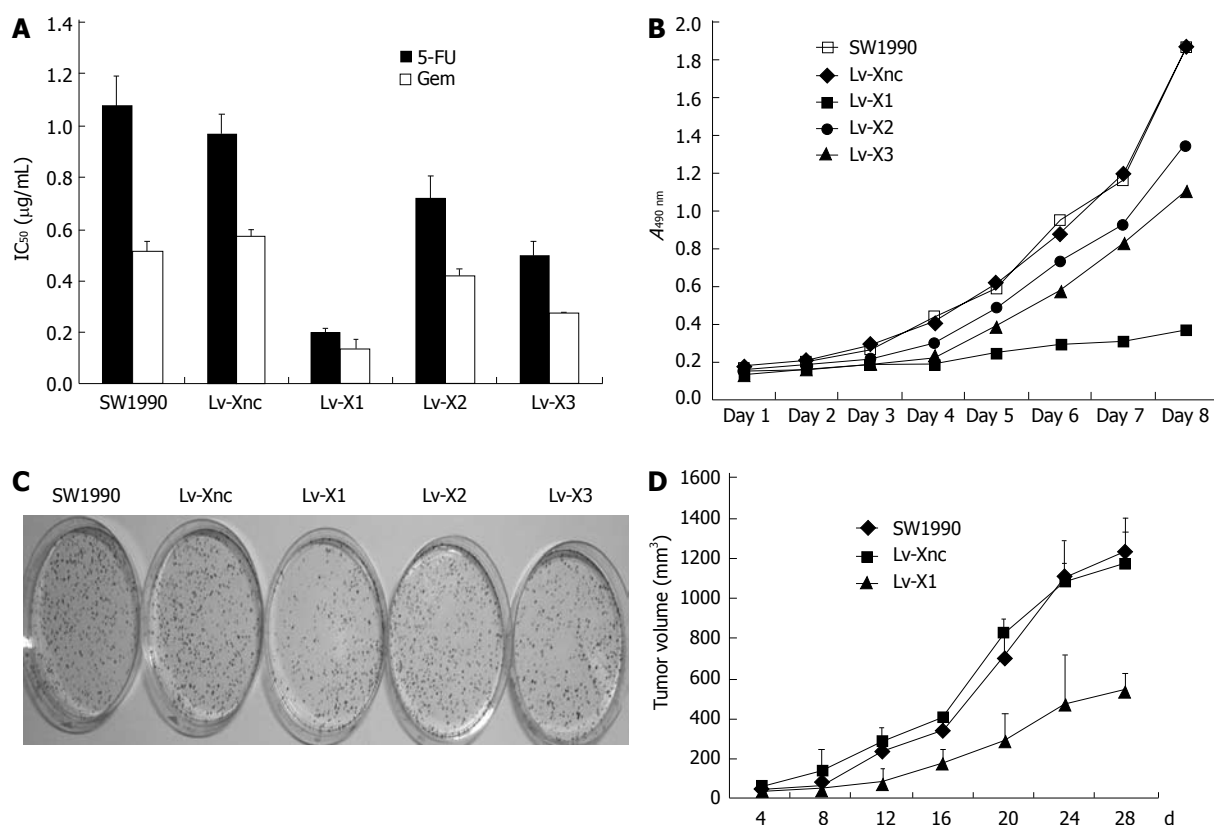


Figure 2 Lentivirus-mediated shRNA targeting the X-linked inhibitor of apoptosis protein gene inhibits the growth of human pancreatic cancer SW1990 cells *in vitro* and *in vivo* and promotes chemosensitivity to drugs. A: Lv-X1, Lv-X2, Lv-X3 promoted 5-fluorouracil (5-FU) or gemcitabine-induced cytotoxicity. The IC₅₀ was determined by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay after exposure to 5-FU or gemcitabine for 72 h; B: The growth curves of different stably transfected cell lines; C: Results of colony formation assay. Lv-X1 had much fewer colonies than Lv-Xnc and untransfected cells. These experiments were performed 3 times; D: SW1990, Lv-Xnc and Lv-X1 cells growth in BALB/c nude mice ($P < 0.05$, from day 12 to day 28).

10 μg/mL 5-FU, respectively; 20.26 ± 0.96-fold, 36.62 ± 3.77-fold at 0.1, 1 μg/mL gemcitabine, respectively), but was unaffected after transfection of Lv-Xnc control. In addition, blank Lv-X1 (1.07 ± 0.03-fold) and Lv-Xnc (1.03 ± 0.01-fold) caspase-3/7 activity, although slightly increased, were not significantly different compared with SW1990 control (Figure 3A), which was also confirmed by DAPI staining and flow cytometry (FCM) (Figure 3B and C). We used DAPI staining to observe the morphological changes of apoptosis and the apoptosis index of DAPI staining analysis was consistent with caspase-3/7 activity (Figure 3B). To validate the results of DAPI staining, SW1990 and stably transfected SW1990 cells (Lv-Xnc, Lv-X1) were stained with annexin V and PI and analyzed by FCM. Cell apoptosis analysis indicated that downregulation of XIAP was not associated with a significantly increased spontaneous apoptosis rate (there were no obvious differences in apoptosis rates among SW1990 (3.03% ± 0.49%), Lv-X1 (5.06% ± 0.54%) and Lv-Xnc (4.21% ± 0.36%) ($P > 0.05$)), while the apoptosis of Lv-X1 + 5-FU (12.7% ± 0.50%) or Lv-X1 + Gem (13.68% ± 0.56%) was significantly increased compared with SW1990 and Lv-Xnc control cells ($P < 0.05$) (Figure 3C). All these results showed that the lentivirus-mediated inhibition of XIAP expression did not lead to acceleration of the apoptosis of SW1990 cells.

Downregulation of XIAP with Lv-X1 decreased p-Akt levels

We observed that decreased expression of XIAP resulted in inhibition of cell proliferation according to the results of the growth curves and colony formation assay of different stably transfected cell lines (Figure 2B-D). Caspase-3/7 activity, DAPI staining and FCM analysis of Lv-X1 groups although slightly increased, showed no significant differences compared with controls (Figure 3A-C). It is reported that apoptotic pathways in cancer functionally crossover with survival pathways PI3K/Akt, and compared with the Lv-Xnc group, XIAP protein expression levels in cells transfected with Lv-X1 were reduced by 92.55% ± 0.78% ($P < 0.05$), and p-Akt protein expression in Lv-X1 transfection groups was reduced by 94.63% ± 0.32% ($P < 0.05$) (Figure 4). We found that downregulation of XIAP with Lv-X1 decreased p-Akt levels, which might explain the phenomenon.

DISCUSSION

It has been reported that XIAP is overexpressed in many human malignancies such as ovarian carcinoma, laryngeal cancer, esophageal cancer, breast cancer, hepatoma cells, colon cancer, and pancreatic cancer. Upregulated levels of XIAP expression have been correlated with

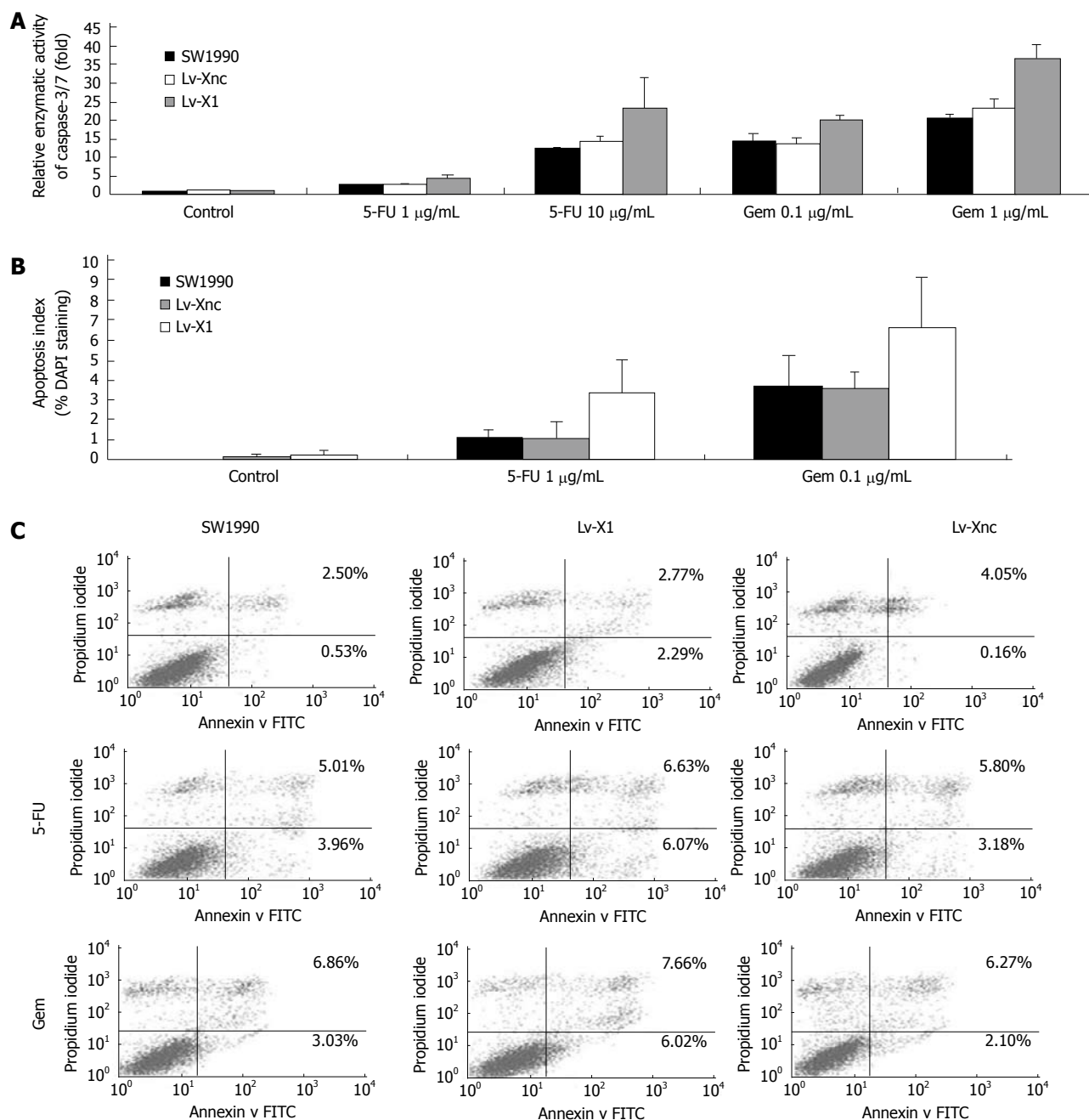


Figure 3 Apoptosis analysis. A: Relative enzymatic activity of caspase-3/7; B: Apoptosis examined by 4',6-diamidino-2-phenylindole (DAPI) staining (200 \times), apoptosis index (%) = Apoptotic cells/Total cells \times 100% (Seen in a same microscopic field and 3 randomized microscopic fields chosen for counting) using DAPI staining. Either gemcitabine or 5-fluorouracil (5-FU) kills cancer cells by inducing apoptosis, and X-linked inhibitor of apoptosis protein knocked out by Lv-X1 can enhance the capacity of the 2 drugs; C: Flow cytometry: The apoptotic rate of Lv-X1 and Lv-Xnc slightly increased but there was no significant difference among SW1990, Lv-X1 and Lv-Xnc. While the apoptotic rate of Lv-X1 + 5-FU and Lv-X1 + gemcitabine increased (12.7% and 13.68%, respectively, $P < 0.05$). FITC: Fluorescein isothiocyanate.

tumor resistance to chemotherapy or radiotherapy, and some researchers have reported that inhibition of XIAP by siRNA, plasmid or adenovirus mediated-shRNA can reduce tumor cell growth, induce apoptosis and enhance the sensitivity of tumor cells to chemotherapeutic or radiotherapeutic agents^[16-21]. Though chemically synthesized siRNA can be introduced into cells *via* traditional delivery strategies including liposomes, polyethyleneimine, or electroporation, these methods have short gene silencing effects and the effects cannot be passed to cell progeny. Other gene delivery systems such as adenovi-

rus showed transient expression of the transgene and immunogenicity. Lentivirus vectors were developed to overcome these disadvantages. Recently, the lentivirus vector-mediated gene therapy including RNAi or overexpression has shown great promise in pancreatic cancer and other tumors because of its long-term gene expression and high efficiency in transducing dividing and non-dividing cells^[22-25].

As we know, Panc-1 and Mia-paca2 are poorly differentiated; SW1990 and Bxpc-3 are moderately differentiated. In our study, human pancreatic cancer cell lines

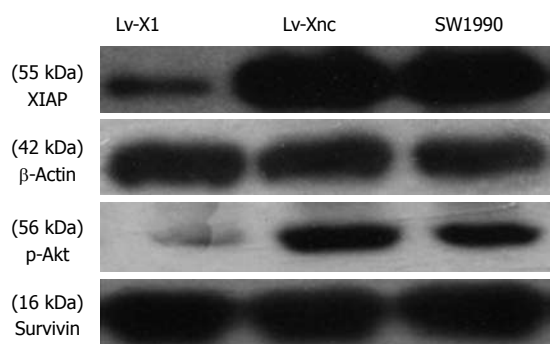


Figure 4 Immunoblot analysis for p-Akt in SW1990 cells after stable transfection. Downregulation of X-linked inhibitor of apoptosis protein with Lv-X1 largely decreased p-Akt protein levels and survivin was not affected (6 mo in culture).

with higher levels of XIAP protein displayed greater 5-FU or gemcitabine chemoresistance (Figure 1A and B).

In this study, we found that lentivirus-based vectors were extremely efficient in transducing SW1990 cells. *In vitro*, 79% of the cells expressed GFP at a MOI of 50. In addition, GFP expression was stable up to 6 mo in culture (data was not shown). To determine whether lentivirus-mediated shRNA could stably inhibit the expression of XIAP, pooled clones were expanded, and stably transfected cells were lysed for Western blotting analysis 6 mo later. The results showed that the expression of XIAP in Lv-X1 cell was still markedly reduced (Figure 4). It indicated that lentivirus-based shRNA resulted in a persistent gene knockdown instead of a transient inhibitory effect.

Furthermore, the strong inhibition of XIAP by lentivirus could inhibit proliferation of SW1990 cells (Figure 2B-D), enhance drug-induced apoptosis and promote chemosensitivity to chemotherapeutic drugs, which also were reported in other studies (Figure 3A-C)^[26,27], and some drugs induce apoptosis through downregulation of cell survival proteins and upregulation of death receptors *via* the reactive oxygen species-mediated upregulation of the CHOP (CCAAT/enhancer-binding protein-homologous protein) pathway^[28]. Some studies showed that silencing the XIAP gene resulted in a significant induction of apoptosis in pancreatic cancer cells Mia-paca2 and AsPC-1^[29]. In this study, we did not find that downregulation of XIAP was associated with a significantly increased spontaneous apoptosis rate (Figure 3A-C), which was consistent with some other previous studies^[30]. To validate the specificity of RNAi targeting XIAP and to avoid off-target phenomenon, we transfected the cells with lentivirus carrying 3 different shRNA sequences against XIAP and determined the level of another IAP family protein survivin (Figures 1-4). The results showed that survivin was not affected by any constructed lentivirus, which was not consistent with previous studies^[31].

One of the greatest challenges for researchers in new treatment strategies for pancreatic cancer is the obvious need to test them in preclinical *in vivo* animal models that have a good probability of being predictive of similar activity in humans. The most used models are xenografts

of human tumors grown subcutaneously in immunodeficient mice such as BALB/c nude mice^[32]. In this study, we determined whether SW1990 cells stably transfected with lentivirus have reduced tumorigenicity. In the xenograft model, BALB/c nude mice that received injection of Lv-X1 cells developed tumors of a smaller size compared with control, and also showed higher levels of XIAP protein and greater tumorigenicity (Figure 2D). These findings were consistent with some previous studies in xenograft models, in which tumors from the parental pancreatic carcinoma cells with stable XIAP knockdown clones showed growth retardation^[33].

Akt, which has been shown to have both prosurvival and antiapoptotic functions, is a serine/threonine kinase that is known to have at least 3 isoforms (Akt1, Akt2 and Akt3), all of which are activated by phosphoinositide 3-kinase (PI3K), and p-Akt is an activity form of Akt. It is reported that total Akt (phosphorylated + non-phosphorylated) was not altered by XIAP, nor was the expression of the p85 subunit of PI3K, suggesting a direct influence of XIAP upon Akt activation rather than an upregulation of Akt expression^[34]. One of the Akt substrates identified to have antiapoptotic effects is Bad, which is the proapoptotic Bcl-2 family member that initiates apoptosis *via* binding antiapoptotic Bcl-2 family members and results in the release of cytochrome c from mitochondria. Akt has also been shown to directly phosphorylate and inactivate caspase-9^[10,35], which is coordinated through binding to XIAP. Recently, XIAP was added to the list of Akt substrates. Akt has been shown to prevent the ubiquitination and degradation of XIAP *via* phosphorylation both *in vitro* and *in vivo*, and XIAP is a downstream target of Akt and a potentially important mediator of the effect of Akt on cell survival^[36]. XIAP is also thought to promote Akt activity, XIAP acting as an E3 ubiquitin ligase for PTEN and promotes Akt activity by regulating PTEN content and compartmentalization, while XIAP silencing reduces constitutive mono- and poly-ubiquitination of PTEN, increases PTEN protein levels, and prevents nuclear accumulation of PTEN^[37]. Furthermore, it has been reported that suppression of XIAP by either siRNA or antisense adenovirus of XIAP induced apoptosis and inhibited Akt-stimulated cell survival in ovarian cancer cells and uterine cancer cells^[11,34]. These results are significant because they suggest a feedback regulation of XIAP and Akt. In this study, we found stable downregulation of XIAP with Lv-X1 in SW1990 pancreatic cancer cells markedly decreased the p-Akt protein level (Figure 4). Perhaps XIAP is a potentially important mediator of the effect of Akt on cell survival in pancreatic cancer cells as well as in ovarian and uterine cancer cells. While XIAP upregulates Akt phosphorylation and requires Akt for its function, so stable downregulation of XIAP markedly decreases the p-Akt protein level in SW1990 pancreatic cancer cells. Thus, it appears that there is an intricate, coordinated regulatory system at play between XIAP and the PI3K/Akt signaling pathway. Additional studies are necessary

to determine the precise molecular mechanism by which XIAP regulates the Akt survival pathway.

In summary, our findings demonstrate for the first time that suppression of XIAP expression *via* lentivirus-mediated shRNA represents a novel strategy for chemosensitizing pancreatic cancer cells to chemotherapeutic drugs. Our study indicates that lentivirus-mediated inhibition of XIAP is an attractive therapeutic strategy in the treatment of pancreatic cancer and justifies the use of lentivirus in cancer gene therapy studies. However, emergence of replication competent lentivirus *in vivo*, transcriptional targeting affected by the chromosomal integration site and risk of oncogene activation by the lentivirus are current problems, and an effective and safe protocol should be developed. Thus, there remains a long road before lentivirus-mediated shRNA targeting XIAP can be introduced into clinical use.

COMMENTS

Background

Pancreatic cancer is one of the most aggressive human malignancies with an extremely poor prognosis and a 5-year survival rate of only approximately 5%, partially because of the low possibility of surgical resection and resistance to chemo-radiotherapy.

Research frontiers

It has been reported that X-linked inhibitor of apoptosis protein (XIAP) is overexpressed in many human malignancies. The upregulated levels of XIAP expression have been correlated with tumor resistance to chemotherapy or radiotherapy. Lentivirus vector-mediated gene therapy has great promise in pancreatic cancer because of its long-term gene expression and high efficiency. Thus it is necessary to determine whether lentivirus-mediated shRNA targeting XIAP gene could be exploited in the treatment of pancreatic cancer.

Innovations and breakthroughs

XIAP proteins were found to be differentially expressed among pancreatic cancer cell lines. Downregulation of XIAP by transfection with XIAP shRNA resulted in decreased p-Akt expression. Moreover, it could inhibit the growth of pancreatic cancer cells *in vitro* and *in vivo* and enhance drug-induced apoptosis and promote chemosensitivity to chemotherapeutic drugs 5-fluorouracil and gemcitabine. Results also suggest that inhibition of XIAP and subsequent p-Akt depletion may have an anti-tumor effect through attenuating the ability of cancer cells to survive. Perhaps XIAP is a potentially important mediator of the effect of Akt on cell survival in pancreatic cancer cells. It appears that there is an intricate, coordinated regulatory system at play between XIAP and the phosphatidylinositol 3-kinases/Akt signaling pathway.

Applications

The results suggest that suppression of XIAP expression *via* lentivirus-mediated shRNA represents a novel strategy for chemosensitizing pancreatic cancer to chemotherapeutic drugs. Lentivirus-mediated inhibition of XIAP is an attractive therapeutic strategy in the treatment of pancreatic cancer and justifies the use of lentivirus in cancer gene therapy studies.

Terminology

XIAP: A member of the IAP family, plays an important role in regulating both apoptosis and cell proliferation; Apoptosis or programmed cell death: Disordered apoptosis and abnormal proliferation have been linked to malignancy development and treatment resistance; Akt: Also termed protein kinase B, represents a subfamily of serine/threonine kinases that promotes cellular survival. RNAi: RNA interference, a sequence specific posttranscriptional gene silencing process, which has been extensively used in the study of gene function and gene therapy for cancer.

Peer review

This is an interesting manuscript. The data is solid, but it is still a long way from lentivirus-mediated shRNA targeting XIAP to clinical use.

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