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ORIGINAL ARTICLE

Suppression of *Bcl-xL* expression by a novel tumor-specific RNA interference system inhibits proliferation and enhances radiosensitivity in prostatic carcinoma cells

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Abstract *Bcl-xL*, a novel member of anti-apoptotic *Bcl-2* family that play important roles in regulating cell survival and apoptosis, is frequently overexpressed in various kinds of human cancers, including prostatic carcinoma. To explore its possibility as a therapeutic target for prostatic carcinoma, we developed a novel tumor-specific RNA interference system by using survivin promoter and employed it to suppress exogenous reporters (*LUC* and *EGFP*) and endogenous gene *Bcl-xL* expression and analyzed its phenotypes. We found that expression of exogenous reporters (*LUC* and *EGFP*) was specifically inhibited in tumor cells but not in normal cells. We also observed that the specific inhibition of *Bcl-xL* in human prostatic carcinoma cells (PC3) strongly suppressed in vitro cell proliferation and in vivo tumorigenicity. We observed significant apoptosis induction and radiosensitivity enhancement in PC3 cells by the RNA interference-mediated suppression of *Bcl-xL* expression. All these results indicate that inhibition of *Bcl-xL* expression can result in potent antitumor activity and radiosensitization in human prostatic carcinoma.

Keywords Survivin promoter · *Bcl-xL* · RNA interference · Inhibitors of apoptosis · Gene therapy · Radiosensitivity · Prostatic carcinoma · PC3

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Introduction

Increased resistance to apoptosis is a hallmark of many tumor cells. The functional inhibition of specific anti-apoptotic factors may provide a rational basis for the development of new therapeutic strategies in cancers [1, 2]. *Bcl-xL* is a novel member of *Bcl-2* family and its over expression has been found in a variety of cancers including human prostatic carcinomas and the inhibition of this molecule is associated with decreased tumorigenesis and resistance to conventional chemotherapy [3–5]. In this study, we investigated the effects of *Bcl-xL* on cell proliferation and explored its possibility as a highly promising molecular target for radiosensitization of human prostatic carcinoma cell.

RNA interference (RNAi) is a sequence-specific post-transcriptional gene-silencing process that has been proposed as a potential treatment method for cancers [6]. Specific gene silencing can be achieved in a variety of cell systems using chemically synthesized siRNA or DNA vector-based shRNA [7, 8]. Most commonly used promoters to drive shRNA expression are the cytomegalovirus (CMV) Pol II or Pol III promoters, including the U6, H1-RNA and tRNA promoters [9, 10]. However, the use of these promoters to drive shRNA expression in vivo would silence a given gene in all cell types, and thus produce undesirable effect in non-target cells. Cell- or tissue-specific property is an important issue to consider in RNAi therapy. Recently, cell-specific gene silencing has been achieved by using cell-specific promoters (SP-C) to drive shRNA system, and a loxP-CRE RNAi regulatable RNAi system has also been developed [11–13].

In the present study, we attempted to develop a tumor specific RNAi system directly under the control of survivin

promoter and explore whether this system could inhibit the expression of exogenous reporter genes [firefly luciferase (*LUC*) and enhanced fluorescent protein (*EGFP*)] in a tumor-specific manner. We constructed corresponding shRNA vectors driven by survivin promoter and demonstrated specific and efficient silencing of targeted genes in tumor cells but not in normal cells in vitro. We also established human prostatic carcinoma PC3 cells stably expressing shRNA targeting *Bcl-xL*, and those cells displayed minimal levels of *Bcl-xL* expression and showed a marked increase in radiosensitivity besides an obvious inhibition of cell proliferation and a notable induction of cell apoptosis. Together, survivin promoter-driven RNAi targeting *Bcl-xL* will be explored as a potential tool for radiosensitization of human prostatic carcinoma cells with tumor specificity and high efficacy.

Materials and methods

Plasmid construction

To construct a survivin promoter-driven shRNA vector (pS-SP-shRNA), the survivin promoter gene (GenBank NM U75285) including 980 bp upstream from the transcription initiation site was amplified from our previously obtained survivin promoter plasmid by polymerase chain reaction (PCR). The PCR product was then subcoloned into the *Bgl*II and *Eco*RI site of pSUPER.retro vector (Ambion, USA) to replace H1-RNA promoter. A minimal poly(A) sequence (5′-TTATTTCCTAGAAAATAAAAGTAACC TAGACACACAACCAAAAAACATACGCCGGCGA-3′) was used as a terminal sequence. shRNAs targeting *LUC, EGFP*, *Bcl-xL* gene (GenBank NM Z23115) and an unrelated shRNA negative control (NC) were designed and synthesized as follows: sh*LUC*, shEGFP, shBcl-*xL* and shControl (see Fig. 1). All of the above sequences were inserted into the *Hind*III and *Bgl*II enzyme sites of pSUPER.retro vector, respectively. Name designation of the recombinant plasmids was pS-SP-sh*LUC*, pS-SP-sh*EGFP*, and pS-SP-shControl. To facilitate the analysis of shRNA efficacy, sv40 promoter-driven luciferase-expressing cassette from pGL3-promoter vector was separately inserted into pS-SP-sh*LUC* and pS-SP-shControl vector between *Eco*RI and *Nhe*I restriction sites. The CMV promoter-driven EGFP-expressing cassette from pGL3-CMV promoter-*EGFP* vector we have constructed previously was separately inserted into pS-SP-sh*EGFP* and pS-SP-shControl vectors between *Eco*RI and *Nhe*I restriction sites. The method used in this research was identical with the one described by Zeng and Huang et al. [14, 15]. The recombinant vectors were confirmed by the digestion analysis of restriction endonuclease and all inserted sequences were verified by DNA sequencing.

Cell culture and transfection

Three human tumor cell lines: PC3, Hep-2, LoVo and normal human prostatic epithelial cell line (BPH1) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in humidified 5%CO_2/95% atmosphere at 37°C. The cell transfection was performed in opti-MEM with the transfection reagent LipofectAMINE PLUS (Invitrogen) following the manufacturer's protocols. Forty-eight hour later after transfection, cells were harvested and passaged into four wells, and colonies stably expressing shRNA were selected with 8 μg/ml puromycin (Sigma, USA). Names of the stably transfected PC3 cells were PC3-s [transfected with pS-SP-sh(*Bcl-xL*)], PC3-NC (transfected with pS-SP-NC) and PC3-pS.retro (transfected with pS.retro parental vector), respectively.

Luciferase assay

PC3, Hep-2, LoVo and BPH1 cell lines were transfected in six-well culture plates with 4.0 μg/well firefly luciferase (Fr-luc)-expressing vectors (pS-SP-sh*LUC*-mpA-sv40-luciferase and pS-SP-shControl-mpA-sv40-luciferase), and Renilla luciferase (Re-luc)-expressing vector (pRL-sv40) was used as transfection controls. The cells were harvested

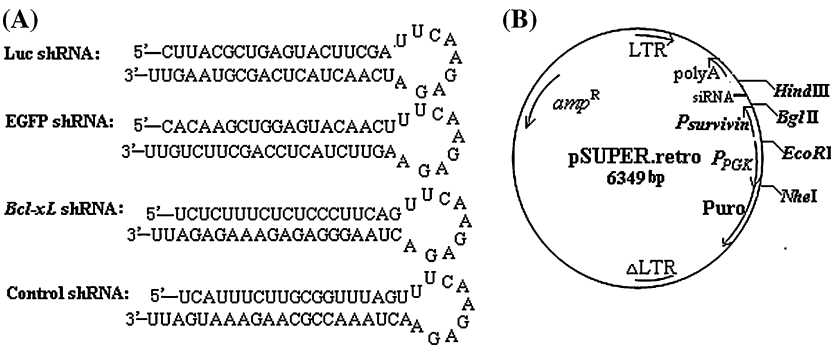

Fig. 1 **a** Predicted structure of small hairpin RNAs and **b** Schematic diagram of the recombinant pSUPER.retro vector. The H1-RNA promoter was replaced by the survivin promoter and shRNA encoding template was inserted between *Hind*III and *Bgl*IIenzyme sites downstream of the survivin promoter

48 h later after transfection. The luciferase activity was measured with a dual luciferase assay Kit (Promega, USA) using a Fluoro and luminometer (Fluoroskan Ascent, FL). The relative luciferase activity was calculated as the ratio of Fr-luc activity to Re-luc activity.

Fluorescence-activated cell sorting analysis

PC3, Hep-2, LoVo and BPH1 cells were transfected in six-well culture plates with pS-SP-sh*EGFP*-mpA-CMV-*EGFP* and pS-SP-shControl-mpA-CMV-*EGFP*, respectively. Seventy-two hours later after transfection, the cells were photographed by using a fluorescence microscope and then harvested. Half of the cells were used to extract total RNA for RT-PCR, the rest of the cells were used to observe the levels of *EGFP* fluorescence using fluorescence-activated cell sorting (FACS Calibur, BD, USA).

Reverse transcription polymerase chain reaction analysis (RT-PCR)

Total RNA was extracted from those stably transfected, untransfected tumor cells and normal BPH1 cells using TRIzol reagent (Invitrogen, USA), respectively, and RNA of 2 Ul (1μg/μl) was used to synthesize cDNA using Superscript First-Strand Synthesis Kit (Promega, USA) following the manufacturer's protocols. The cDNA was used to amplify the survivin, *EGFP* and *Bcl-xL* fragment, while the house keeping gene β-actin was also amplified as an internal standard. The corresponding primer sequences were as follows: survivin, forward: 5′-TTCTCAAGGACCACCG-CATC-3′, reverse: 5′-AGAG GCCTCAATCCATGG-3′; *EGFP*, forward: 5′-TGCCAC CTACGGCA-AGCTGA-3′, reverse: 5′-TCGATGTTGTG GCGGATCTT-3′; *Bcl-xL*, forward: 5′-AGTAATGACT-GTGTAGCACA-3′, reverse: 5′-CATTTCCGACTGAAG AGTg-3′; β-actin, forward: 5′-AGCAACC-GGGAGCTG GTGG-3′, reverse: 5′-CATTTCCGACTGAAGAGTG-3′. The cycling program was performed as follows: 1cycle of 94°C for 3 min; (survivin) 35cycles of 94°C for 40 s, 62°C for 40 s, 72°C for 90 s; (*EGFP*) 30 cycles of 94°C for 40 s, 56°C for 40 s, 72°C for 90 s; (*Bcl-xL*) 35 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 90 s; followed by a final elongation step of 72°C for 10 min. Then RT-PCR products were electrophoresed through a 1.5% agarose gel with ethidium bromide. Signals were quantified by densitometric analysis using the Labworks Image Acquisition (UVP, Inc., Upland, CA, USA). The inhibitory rate of *Bcl-xL* mRNA expression in PC3-s cells was calculated as follows: inhibitory rate = [1– (PC3-s *Bcl-xL* density/PC3-s β-actin density)/(PC3 *Bcl-xL* density/PC3 β-actin density)] × 100%.

Western blot analysis of *Bcl-xL* protein

The PC3-s cells were lysed in 50 μl lysis buffer (1 mM dithiothreitol, 0.125 mM EDTA, 5%glycerol, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1% Triton X-100 in 12.5 mM Tris–HCl buffer, pH 7.0) on ice for 30 min, and the lysates were cleared by centrifugation. Proteins were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membrane, blocked by 5% skim milk, and probed with anti-*Bcl-xL* (Merck, GER) and anti-β-actin (Sigma, USA) antibody. Following incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Amersham Pharmacia Biotech), immunoblots were visualized by chemiluminescence using a chemiluminescence kit (Invitrogen) and the specific bands were recorded on X-ray film. The inhibitory rate of *Bcl-xL* protein expression was calculated as follows: inhibitory rate = [1 – (PC3-s *Bcl-xL* density/PC3-s β-actin density)/(PC3 *Bcl-xL* density/PC3 β-actin density)] × 100%.

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay

The cells (PC3, PC3-pS.retro, PC3-NC and PC3-s) were seeded at a density of 3×10^3 cells/well in 96-well plates in DMEM containing 10% fetal bovine serum at a final volume of 0.2 ml, respectively, and were grown for 7 days at 37°C with 5% carbon dioxide. During this period, we fed those cells every 2–3 days with complete medium, and selected three wells from each group of cells every day at random for methyl thiazolyl tetrazolium (MTT) (60 mg/well) assay. Then, the cells were incubated at 37°C for 4 h, the reaction was stopped by the addition of 200 ml/well of DMSO, lysed for 10 min. The protracted cell growth curve and the results of the inhibitory rates of cell growth were applied to absorbance (*A*) at 490 nm using Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA) as follows: inhibitory rate = (1 – PC3-s *A*/PC3 *A*) × 100%.

Colony formation assay

Approximately 2×10^2 cells transfected with pS-SP-sh(*Bcl-xL*), pS-SP-shControl, pS.retro parental vector were plated in 10-cm culture dishes, respectively. After 18 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

Flow cytometric analysis of apoptosis

An annexin V-fluorescein isothiocyanate apoptosis detection kit (Zymed, USA) was used to detect cell apoptosis.

The cells (PC3, PC3-pS.retro, PC3-NC and PC3-s) were seeded in 100 ml flasks, respectively, and incubated until there was approximately 90% confluence in DMEM containing 10% fetal bovine serum. Then the cells were harvested, washed with ice-cold phosphate buffered saline (PBS) twice and resuspended in binding buffer (10 mM of HEPES, pH 7.4, 150 mM of NaCl, 2.5 mM of $CaCl_2$, 1 mM of $MgCl_2$, 4% bovine serum albumin). Annexin V-fluorescein isothiocyanate (0.5 μg/ml) and propidium iodide (0.6 μg/ml) were then added to a 250 ml aliquot (6×10^6 cells) of this cell suspension. After a 15-min incubation in the dark at room temperature, stained cells were immediately analyzed by EPICS-ELITE-ESP Flow Cytometry (Coulter Biosciences). All of the samples were assayed in triplicate, and the cell apoptosis rate calculated as: apoptosis rate = (apoptotic cell number/total cell number) × 100%.

TdT-mediated dUTP nick end labeling (TUNEL) assay

The stably transfected and untransfected PC3 cells were seeded, respectively, in 100 mm culture plate in which coverslip was put beforehand to allow the seeded cells to grow on. Then, the cells on coverslip were harvested in day 1, 3, 5, 7, respectively, and was used to detect the incorporation of labeled nucleotides to DNA strand breaks by TUNEL assay (Keygen Biotech, Nanjing, China) according to the manufacturer's specifications. The negative control was set up with no TdT enzyme added in the staining process.

Tumorigenicity in mice xenograft model

The effect of *Bcl-xL* on tumorigenicity was assessed by subcutaneous injection of pS-SP-sh(*Bcl-xL*), pS-SP-NC, or pS.retro parental vector stably transfected PC3 cells and untransfected PC3 cells in athymic nude mice. Each aliquot of approximately 2×10^6 cells suspended in 100 μl of PBS containing 20% of Matrigel Growth Factor Reduced (Becton Dickinson Labware, Flanklin, NJ, USA) were injected into 8-weeks-old BALB/c male nude mice which were maintained under pathogen-free conditions. The inoculations were done in six mice for each group. Tumor growth over a period of 4 weeks was monitored and measured. Each tumor volume in mm^3 was calculated by the following formula: $V = 0.4 \times D \times d^2$ (V volume; D longitudinal diameter; d latitudinal diameter). Animal experiments in this study were done in compliance with the Fourth Military Medical University of Medicine institutional guidelines.

Clonogenic survival assay

The appropriate number of stably transfected or untransfected PC3 cells was plated in 60 mm dishes, respectively, and allowed to attach for 6 h, then cells were treated with different doses of 6 MV X-ray radiation by a 23EX accelerator (Varian; the radiation doses were 0, 2, 4, 6, and 8 Gy, respectively; the dose efficiency was 400 cGy/min). The medium was replaced with a fresh one 24 h later after radiation and incubated for another 2 weeks. Colonies were fixed with methanol and stained with Giemsa for about 15 min. Stained colonies that had more than 60 cells were counted and coloning efficiency calculated as: coloning efficiency = (colony number/total cell number) × 100%, in triplicate. Furthermore, the cell survival fraction was counted out and the cell survival curve was drafted by the standard model, $S = 1 - (1 - e^{-D/D_0})^N$ (S cell survival fraction; D: radiation dose; e: the bottom of the natural logarithm; D_0 the mean death dose; N: extrapolate number, D_q standard threshold dose, can be acquired from the cell survival curve). Finally, the radiosensitization ratio was calculated (a ratio of D_0 and a ratio of D_q).

Statistical analysis

Data are expressed as mean ± SE and all statistical analyses were performed by using SPSS10.0. Comparisons among all groups were performed with the one-way analysis of variance (ANOVA) test and Student Newman Keuls method. Differences were considered significant at $P < 0.05$.

Results

Construction of survivin promoter-driven shRNAs-expressing vectors

DNA sequencing results showed that we successfully constructed four different survivin promoter-driven shRNA expression vectors (pS-SP-shRNA) targeting *LUC*, *EGFP*, *Bcl-xL* and the negative control. Those vectors were named pS-SP-sh*LUC*, pS-SP-sh*EGFP*, pS-SP-sh(*Bcl-xL*), and pS-SP-shControl(NC), respectively (Fig. 1).

The level of survivin gene mRNA expression detected by RT-PCR

The level of survivin expression in tumor cell lines was determined by comparing the level of survivin mRNA in three tumor cells (PC3, Hep-2 and LoVo) to that in a normal epithelial cell line (BPH1) by RT-PCR analysis. As shown in Fig. 2, survivin gene was expressed at high levels in three human tumor cells but not detected in human normal cells. This provided a basis for the survivin promoter-driven shRNA to silence targeted genes with tumor specificity and high efficacy.

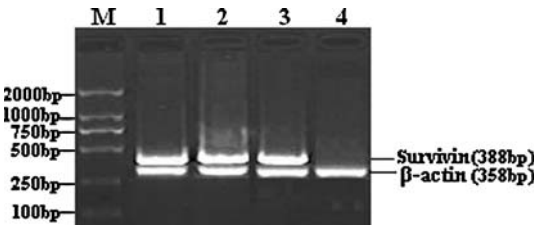

Fig. 2 The survivin gene expression detected by RT-PCR. The high-level expression of survivin gene was detected in three tumor cell lines but not in normal cells. *M* DGL2000; *1* PC3; *2* Hep-2; *3* LoVo; *4* BPH1

Effect of shRNA on exogenous reporter gene expression

First, we investigated whether survivin promoter-driven sh*LUC* could reduce the expression of firefly luciferase. To facilitate the analysis of shRNA efficacy, we constructed the pS-SP-sh*LUC*-sv40 promoter-luciferase (pSsh*LUC*, Fig. 3a) and pS-SP-shControl-sv40 promoter-luciferase (pSshControl) vectors. Cells were transfected with the Fr-luc-expressing vectors (pSsh*LUC* or pSshControl) and the Re-luc-expressing vector pRL-sv40. Luciferase activity was determined by a dual luciferase assay 48 h later after transfection. The relative luciferase activity was defined as the ratio of Fr-luc activity to Re-luc activity. The mean pSsh*LUC*/pSshControl ratios were 0.289 ± 0.051, 0.320 ± 0.042 and 0.346 ± 0.038 in PC3, Hep-2 and LoVo tumor cell lines, respectively ($P < 0.05$; Fig. 3b), while the mean pSsh*LUC*/pS*LUC* ratio was 0.865 ± 0.0924 in BPH1 cell lines.

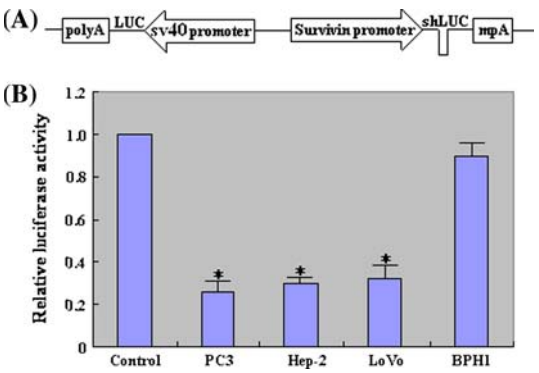

Fig. 3 Reducing luciferase activity by RNAi targeting firely luciferase. **a** Structure of recombinant vector (pS-SP-sh*LUC*-sv40 promoter-*LUC*). **b** sh*LUC* efficacy in three different tumor cell lines (*PC3*, *Hep-2* and *LoVo*) and normal BPH1 cells transfected with pSsh*LUC* and pSshControl (Fr-luc), respectively and the Renilla luciferase (Re-luc)-expressing vector pRL-sv40 served as a transfection control. The relative luciferase activity was defined as the ratio of Fr-luc activity to Re-luc activity. The activity of pSshControl·Re-luc (*control*) was designated as 1.0. Each group contained 5 wells and experiments were performed 5 times. $*P < 0.01$

Second, we explored the inhibitory effects of survivin promoter-driven shRNA targeting another exogenous reporter gene, *EGFP*. We also constructed pS-SP-sh*EGFP*-CMV promoter-EGFP (pSsh*EGFP*; Fig. 4a) and pS-SP-shControl-CMV promoter-(pSshControl) vectors. The level of EGFP mRNA was determined by RT-PCR, normalized to the level of β-actin mRNA. Results showed that compared with controls, the level of EGFP mRNA decreased in PC3, Hep-2 and LoVo cells transfected with pSsh*EGFP*, but there was no obvious change in BPH1 normal cells (Fig. 4b).

The inhibitory effects of sh*EGFP* on EGFP protein expression were assessed by fluorescence microscopy and FACS. The relative level of *EGFP* fluorescence had no obvious change in BPH1 cells, but decreased markedly in PC3, Hep-2 and LoVo cells transfected with pSsh*EGFP* as determined by direct visualization under a fluorescence microscope (Fig. 4c) and FACS analysis ($P < 0.01$; Fig. 4d). The results were in accordance with those at the mRNA level.

Significant downregulation of *Bcl-xL* gene expression by shRNA

Compared with untransfected PC3 cells, the expression levels of *Bcl-xL* mRNA and protein in PC3-s cells decreased significantly, and the inhibitory rates were 81.7 and 58.6% for PC3-s, respectively, while there were no significant differences in the levels of *Bcl-xL* mRNA and protein expression among PC3-NC, PC3-pS.retro and untransfected PC3 cells ($P > 0.05$). The expression levels of β-actin mRNA and protein were nearly equal among in all groups of cells ($P > 0.05$; Fig. 5a, b). These results show that the survivin promoter can efficiently drive siRNA expression and the siRNA sequence targeting *Bcl-xL* is effective in downregulating *Bcl-xL* expression.

Inhibtion of in vitro cell proliferation and colony formation by downregulation of *Bcl-xL* expression

To analyze the changes of phenotype, we first investigated *Bcl-xL* siRNA on the proliferation of PC3 cells in vitro. In this experiment, cellular proliferations were monitored by MTT assay daily for 7 days. The cell growth curve showed that compared with untransfected PC3 cells, PC3-s proliferation was significantly inhibited in a time-dependent manner and the highest inhibitory rate was 56.4 ± 3.4% on day 7. ($F = 2513.163$, $P < 0.05$), while PC3-NC and PC3-pS.retro cells showed no significant inhibition of the proliferation ($P > 0.05$; Fig. 6). In colony formation assay, as expected from the results of MTT assay, the numbers of

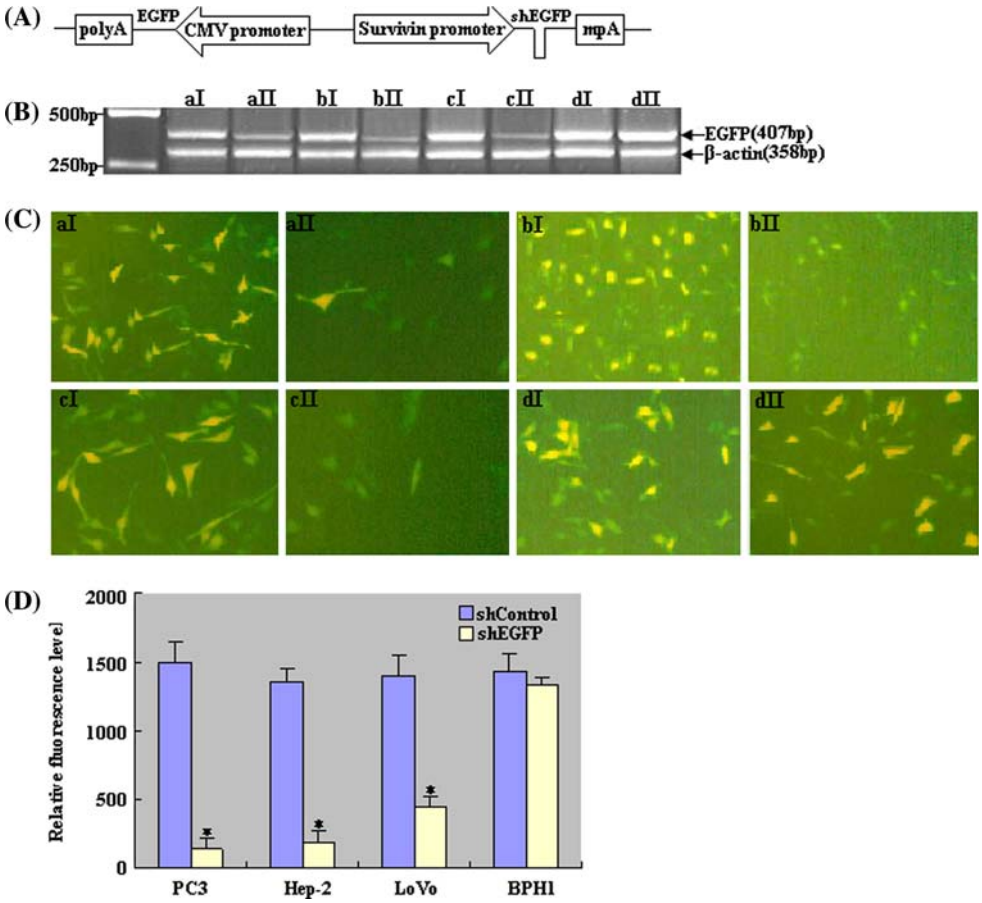

Fig. 4 Inhibition of *EGFP* expression by RNAi targeting *EGFP*. **a** Structure of recombinant vector (pS-SP-sh*EGFP*-CMV promoter-*EGFP*). **b** RT-PCR analysis of *EGFP* mRNA expression. PC3, Hep-2, LoVo and BPH1cells were transfected with pSsh*EGFP* and pSshControl, respectively. Lanes: a, PC3; b, Hep-2; c, LoVo; d, BPH1; *I* cells expressing shControl; *II* cells expressing shEGFP. **c** The levels of *EGFP* expression detected by fluorescence microscopy (×200). Cells were photographed 72 h later after transfection with pSshControl or pSsh*EGFP*. The cells and cell transfection panel are the same as (**b**). **d** The relative levels of *EGFP* fluorescence detected by FACS. $^{*}P < 0.01$

colonies were obviously decreased in PC3-s compared with untransfected PC3 cells (Fig. 7). All results suggest that suppressing *Bcl-xL* expression by survivin promoter-driven shRNA vector can lead to significant inhibition of prostatic carcinoma cell proliferation in vitro.

Downregulation of *Bcl-xL* expression inhibits tumorigenicity in vivo

We wondered whether the inhibition of *Bcl-xL* expression in prostatic cancer cells would affect their ability to develop tumors in nude mice. To address this question, we established stable transfectants of PC3-s, PC3-NC and PC3-pS.retro cells. Next, we injected the aliquot of 2×10^6 above cells and untransfected PC3 cells s.c. into each group of six athymic nude mice, respectively, and monitored their tumor growth. As shown in Fig. 8, the PC3-NC, PC3-pS.retro and untransfected PC3 cells all gave rise to tumors within 4 weeks in all groups of six mice, whereas the PC3-s cells did not develop visible tumors in any of them. These results indicated that RNAi-mediated downregulation of *Bcl-xL* expression exerted a strong antitumorigenic effect in vivo on prostatic cancer cells.

Analysis of apoptosis

Cell apoptosis analysis by flow cytometry indicated that the apoptosis rate of PC3-s significantly increased to 33.5 ± 1.2% compared with the apoptosis rates of PC3-NC, PC3-pS.retro and PC3 blank cells ($P < 0.05$, respectively), while there were no differences in apoptosis rates among the later three kinds of cells ($P > 0.05$; Fig. 9a), which was 6.4 ± 1.1%, 5.1 ± 0.8% and 4.8 ± 0.7%, respectively. TUNEL assay based on labeling of break DNA strand to detect the apoptosis cells showed that a dark brown DAB signal indicating positive staining was notably observed in PC3-s cells, but not in PC3-NC, PC3-pS.retro and untransfected PC3 cells at day 7 after transfection (Fig. 9b). All these results showed that the RNAi-mediated inhibition of *Bcl-xL* expression could lead to accelerate the apoptosis of human prostatic carcinoma cells.

Bcl-xL gene RNAi increases the radiosensitivity of PC3 cells

Compared with untransfected PC3 cells, coloning efficiency decreased notably in PC3-s at the same dose of

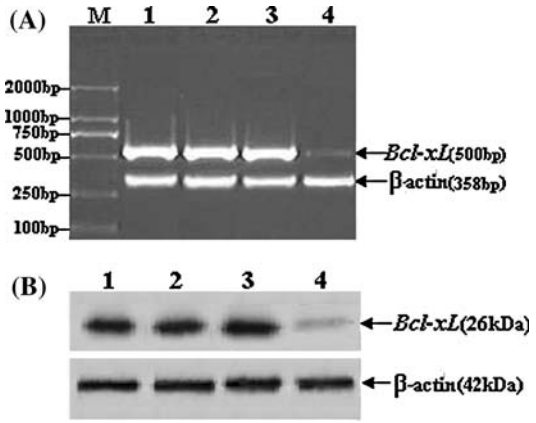

Fig. 5 Inhibition of *Bcl-xL* expression in PC3 cells by RNA interference. **a** mRNA expression of *Bcl-xL* detected by semi-quantitative RT-PCR. Total RNA from PC3, PC3-pS.retro, PC3-NC, PC3-s cell lines was reverse transcribed and amplified by the primers of *Bcl-xL* and *β*-actin, respectively. The products of PCR were separated on a 1.5% agarose gel. Densitometric analysis was performed using the Labworks Image Acquisition and the inhibitory rate of *Bcl-xL* mRNA expression in PC3-s was 81.7%. **b** Protein expression of *Bcl-xL* detected by Western blotting. Membranes were probed with antibodies for targeted protein, and expression levels were normalized for loading by probing for *β*-actin. The blots were developed using a chemiluminescence (Invitrogen) kit. Densitometric analysis was performed using the Labworks Image Acquisition and the inhibitory rate of *Bcl-xL* protein expression in PC3-s was 58.6%. *M* DGL2000 marker; *1* PC3; *2* PC3-pS.retro; *3* PC3-NC; *4* PC3-s

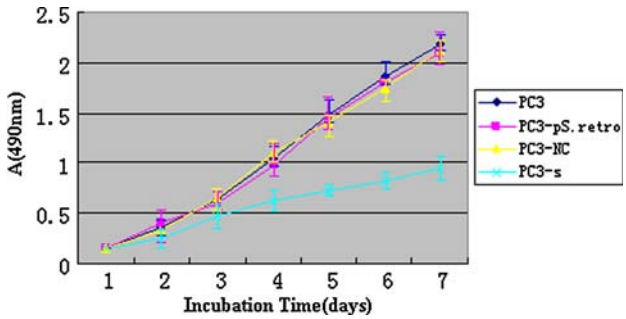

Fig. 6 Cell proliferation detected by MTT. The data in each time point are averaged values from six replicates. Cell proliferation was inhibited notably in a time-dependent manner, and the highest inhibitory rate was 56.4 ± 3.4% ($P < 0.05$) on day 7

radiation ($P < 0.05$; Fig. 10; Table 1); the cell survival curve showed a significant decrease in D_0 and D_q too, which were 3.28 and 1.24, respectively ($P < 0.05$), and the radiation enhancement ratios were 2.08 (a ratio of D_0) and 1.76 (a ratio of D_q; Fig. 11; Table 2). There were no obvious differences in coloning efficiency among PC3-NC, PC3-pS.retro, and untransfected PC3 cells ($P > 0.05$; Fig. 10; Table 1), their D_0 and D_q were similar, and the radiation enhancement ratios were close to 1 (Fig. 11; Table 2).

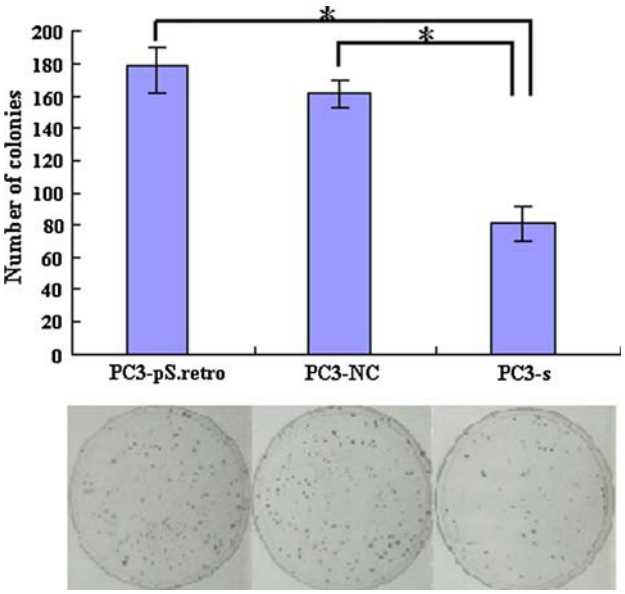

Fig. 7 Colony formation assay results. The PC3-s cells showed much less colonies than PC3-pS.retro and PC3-NC cells. These experiments were performed for four times. * $P < 0.05$

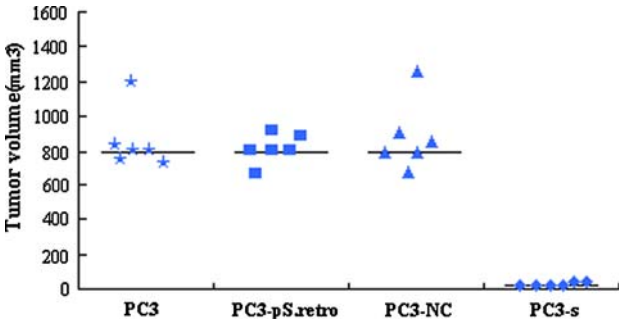

Fig. 8 Inhibiting in vivo tumorigenicity by stable downregulation of *Bcl-xL* expression. Tumorigenicity of the stably transfected PC3 cells and untransfected PC3 cells in the mouse-xenografted model. Six mice were included in each group. The stably transfected PC3 cells were inoculated s.c. into all six nude mice. Sizes of the tumors were measured at 4 weeks after inoculation

Discussion

RNAi is a process of sequence-specific post-transcriptional gene silencing initiated by dsRNA [16]. Usually, the vector based siRNA delivery is composed of a promoter, a short cDNA sequence encoding siRNA and a transcription termination sequence. Most commonly used promoters to drive siRNA expression are CMV promoter and Pol III promoters, including U6, H1 and tRNA promoters [9, 10], which direct high levels of siRNA expression but do not possess cell specificity. Utilization of a tumor-specific promoter that is predominantly active in tumor cells would be an ideal strategy for restricting therapeutic gene expression.

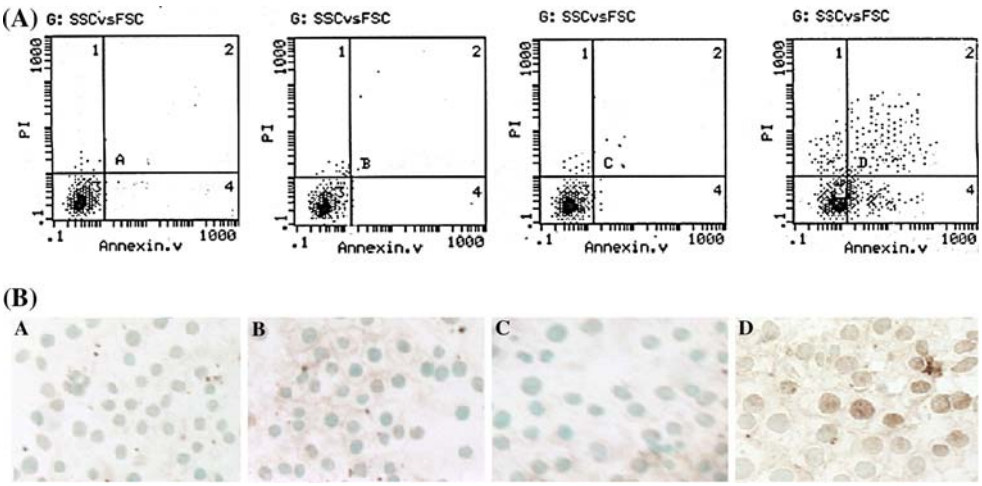

Fig. 9 Cell apoptosis detected by flow cytometry and TUNEL assay. **a** The apoptotic rate of PC3-s obviously increased (33.5 ± 1.2%, $P < 0.05$); while there were no significant differences in cell apoptosis among PC3-NC, PC3-pS.retro, and PC3 cells ($P > 0.05$). **b** Stably transfected and untransfected PC3 cells stained by TUNEL method with dark brown positive signal located in nucleus. A. PC3; B. PC3-pS.retro; C. PC3-NC; D. PC3-s

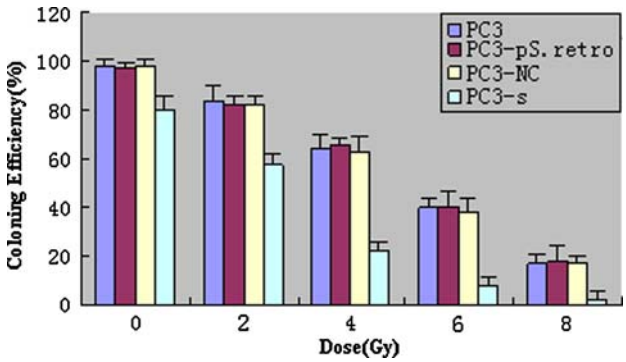

Fig. 10 Comparison of coloning efficiency at different radiation doses. At the same dose of radiation, coloning efficiency declined notably in PC3-s, compared with untransfected PC3 cells

Survivin is a member of the IAP family and it is overexpressed in most tumor cells but not in normal cells, just as shown in Fig. 2, so transcriptional activity of the survivin promoter displays high specificity in tumor cells [17, 18]. Recent reports showed that the survivin promoter might be a potential candidate for targeted cancer gene therapy [19]. To explore the possibility of the survivin promoter used to drive shRNA expression vector and silence gene expression in a tumor-specific manner, we constructed the survivin promoter-driven shRNA vector in the form of a small hairpin structure followed by a minimal polyA(mpA). Our experiment data showed that survivin promoter-driven shRNA could inhibit the expression of the exogenous reporter genes (firely luciferase and *EGFP*) in cancer cells (PC3, Hep-2 and LoVo) but not in normal cells (BPH1). BPH1 cells are normal human prostatic epithelial cells without endogenous survivin expression. Survivin promoter-driven sh*LUC* and sh*EGFP* reduced both luciferase expression and *EGFP* expression (mRNA and protein) by about 70–80% level. This suggests that survivin promoter-driven shRNA can inhibit targeted gene expression with tumor specificity and high efficacy.

Consequently, we investigated the possibility of survivin promoter-driven shRNA exerting effects on the endogenous gene *Bcl-xL* expression. *Bcl-xL*, a member of the *Bcl-2* family, is overexpressed in various types of cancer cells including prostatic carcinoma [20–22]. The overexpression of *Bcl-xL* leads to resistance to chemotherapeutic drugs and radiotherapy. Depletion of *Bcl-xL* by shRNA, antisense cDNA, or antisense oligodeoxynucleotides can impair tumor cell growth, sensitize cells to apoptosis-inducing agents, and increase cell sensitivity to chemotherapy [23, 24]. In our study, we downregulated the expression of *Bcl-xL* mRNA and protein in the human prostatic carcinoma cell PC3 by survivin promoter-driven shRNA, which inhibited transfected PC3 cell growth and colony formation ability. The apoptosis detection by FCM and TUNEL assay showed positive results, which explained the in vivo abrogation of tumorigenicity in mouse xenograft model. All of these data maybe have laid a foundation for further tumor therapeutic investigation.

Table 1 Coloning efficiency at different radiation doses

Cell	Coloning efficiency (mean ± SD%)				
	0 Gy	2 Gy	4 Gy	6 Gy	8 Gy
PC3	98.20 ± 1.15	83.42 ± 1.03	64.26 ± 2.58	40.23 ± 2.16	17.34 ± 1.36
PC3-pS.retro	97.01 ± 2.32	81.89 ± 1.47	65.45 ± 2.18	39.86 ± 1.86	18.26 ± 1.28
PC3-NC	97.82 ± 2.08	82.36 ± 1.53	63.06 ± 1.14	37.61 ± 2.12	16.92 ± 2.24
PC3-s	79.83 ± 1.26*	56.84 ± 1.39*	22.36 ± 0.87*	8.25 ± 1.66*	2.27 ± 0.68*

Comparison with untransfected PC3 cells

* $P < 0.05$

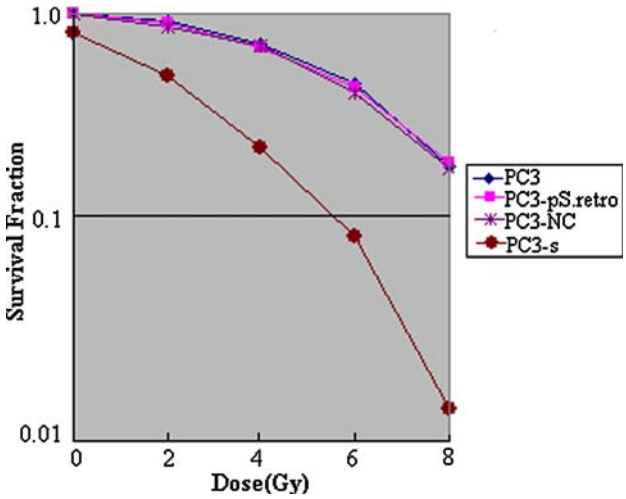

Fig. 11 Comparison of coloning efficiency at different radiation doses**.** There was a significant decrease in D_0 and D_q, which were 3.28 and 1.24, respectively ($P < 0.05$), and the radiation enhancement ratios were 2.08 (a ratio of D_0) and 1.76 (a ratio of D_q)

Table 2 Related parameters of cell survival curve standard model

Cell	D_0	D_q	N	Ratio of D_0	Ratio of D_q
PC3	6.63	2.42	6.22		
PC3-pS.retro	6.56	2.38	6.41	0.98	1.01
PC3-NC	6.46	2.36	6.22	1.03	1.02
PC3-s	3.28*	1.24*	3.40	2.08	1.76

Comparison with untransfected PC3 cells

* $P < 0.05$

Prostatic carcinoma is the most common nondermatologic cancer and radiotherapy is an important therapeutic tool for its treatment [25]. With the increased incidence of prostatic carcinoma, radiotherapy should be attached more importance to. Several studies indicated that knockdown of *Bcl-xL* expression by AS-ODN or small interefercing RNA (siRNA) could enhance cell radiosensitivity in cholangiocarcinoma, cervical carcinoma and pancreatic cancer via caspase-dependent or independent pathway [26]. To investigate whether the overexpression of *Bcl-xL* works on radiosensitivity in PC3 cells, we suppressed *Bcl-xL* expression by RNAi and compared the changes of radiosensitivity before and after transfection with clonogenic survival assay. Results showed that the inhibition of *Bcl-xL* expression might enhance radiosentivity. The radiosensitization mechanism may be concerned with its effect on cell inhibition and apoptosis just as described previously in this report or the ability of DNA break repair, but the accurate mechanism need further study.

In conclusion, survivin promoter-driven shRNA expression could effectively and specifically suppress gene expression in different tumor cells but not in normal cells. Our results also showed that inhibition of *Bcl-xL* expression with this system could lead to cell proliferation inhibition, apoptosis induction and obvious radiosensitivity enhancement in human prostatic carcinoma cells. This system might be a potential approach of the radiosensization of human prostatic carcinomas in the future.

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