Knockdown of survivin expression by siRNAs enhances chemosensitivity of prostate cancer cells and attenuates its tumorigenicity

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Survivin, a member of inhibitor of apoptosis family protein, has become an attractive therapeutic target in cancer due to its selective expression in tumor cells and its important roles for tumor cell viability. Here, we show that vector-based small interfering RNAs (siRNAs) silenced survivin expression in prostate cancer cells, resulting in significantly reduced cell proliferation and enhanced apoptosis, and increased the sensitivity of prostate cancer cells (PC-3) to the apoptosis-inducing agent, platinol. Furthermore, PC-3 cells transfected with the siRNA-expressing vector showed lower tumor formation in nude mice xenografts in vivo. These results demonstrated that inhibition of survivin expression by siRNA attenuated the malignant phenotypes of prostate cancer cells, and may provide a novel approach for gene therapy of androgen-independent prostate cancer.

Keywords RNA interference; survivin; prostate cancer; apoptosis; gene therapy

Received: August 16, 2008 Accepted: December 12, 2008

Introduction

Increases in the susceptibility to chemotherapy would represent an important advancement in the treatment of patients with advanced and metastatic prostate cancer [1]. Survivin is a 16.5 kDa intracellular protein with 142 amino acid residues and belongs to the inhibitor of apoptosis (IAP) family [2]. It is a bifunctional member of the IAP family that counteracts cell death and controls mitotic progression [3]. Survivin is present during embryonic development, but becomes undetectable in most adult tissues [4]. In nearly all human cancers examined so far, survivin expression is re-activated [5–7]. Predictive and prognostic significances of survivin have been implemented in some types of cancer [8–13]. In general, two considerations have made survivin an attractive therapeutic target in cancer, namely, its selective expression in tumor cells and its important roles for tumor cell viability [14].

In recent years, considerable efforts have been made to develop strategies for modulating apoptosis in cells of cancer and other human diseases [15]. In this context, approaches to counteract survivin in tumor cells have been proposed, with the dual aims to inhibit tumor growth through an increase in spontaneous apoptosis and to enhance tumor cell responsiveness to apoptosis-inducing agents [16]. To date, different kinds of survivin antagonists, including antisense oligonucleotides, ribozymes, small interfering RNAs (siRNAs), and dominant-negative mutants, as well as cyclin-dependent kinase inhibitors, have been tested [17].

RNA interference (RNAi) is a genetic interference phenomenon directed by double-stranded RNA. RNAi specifically and efficiently degrades homogeneous mRNA, resulting in post-transcriptional gene silencing [18,19]. Since its discovery in 1998, RNAi has been rapidly developed into one of the most widely applied technologies with therapeutic potential in molecular and cellular research [20,21].

To explore the feasibility of survivin-targeted siRNAs therapy for prostate cancer, the expression of survivin in androgen-independent prostate cancer cell lines PC-3, PC-3M, and DU145, and androgen-dependent prostate cancer cell lines LNCaP and 22RV1 were investigated. Survivin-targeted siRNAs expression vectors were constructed and androgen-independent prostate cancer cell line PC-3 was transfected with the vector-based siRNA.
Next, the biological effects of survivin knockdown on cell chemosensitivity and tumorigenicity were observed in vitro and in vivo. These experiments lay a substantial foundation for understanding the function of survivin and gene therapy of human androgen-independent prostate cancer.

Materials and methods

Materials
pSilencer3.1-H1neo- and pSilencer3.1-H1neo-negative control vectors were purchased from Ambion Inc. (Austin, USA). RPMI-1640 medium and Trizol reagent were purchased from Gibco-BRL (Life Technologies, Inc., Grand Island, USA). Taq DNA polymerase and dNTPs were obtained from Promega (Madison, USA). DNA marker DL-15000, T4 DNA ligase, BamHI, and HindIII were bought from Takara (Dalian, China). Lipopectamine 2000™ and reverse transcription kit were purchased from Invitrogen (Carlsbad, USA). Polyclonal rabbit anti-human survivin antibodies, polyclonal rabbit anti-β-actin antibodies, and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were purchased from Maxim Biological Co. (Fuzhou, China).

Cell lines and culture
The human androgen-independent prostate cancer cell lines PC-3, PC-3M, and DU145, and androgen-dependent prostate cancer cell lines LNCaP and 22RV1 were maintained in our laboratory and were grown as monolayer in RPMI-1640 media containing 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ incubator.

Immunocytochemical staining
Immunocytochemical staining was performed using the streptavidin biotin complex (SABC) method with an SABC kit (Boster, Wuhan, China). Adherent cells were treated with 3 ml/l H₂O₂ in methanol for 30 min to bleach the endogenous peroxidase activity. Samples were blocked with normal goat serum for 30 min at room temperature, followed by incubating with the rabbit anti-human survivin antibody (1:200) overnight at 4°C. The samples were then incubated with the anti-rabbit IgG antibody and were developed in a substrate solution of 0.1 ml/l diaminobenzidine-hydrogen peroxide, followed by counterstaining with hematoxylin. Tumor cell immunostaining was scored by: the percentage of immunopositive cells (0–100) × staining intensity grades (0, 1, 2, or 3) [22].

Reverse transcription–PCR and calculation of relative survivin mRNA levels
Cellular RNA was isolated from transfected PC-3 cells using the Trizol reagent according to the manufacturer’s instructions. Total RNA (1.0 µg) was reverse-transcribed into cDNA using a reverse transcription kit. The resultant cDNA was amplified using primers for the human survivin: sense, 5'-CTTTCTCAAGGACCGCATCT-3'; and antisense, 5'-GACCTTTCGCGCTTCTT-3', which produced a 359-bp fragment. Cycling conditions were: initial denaturation at 95°C for 2 min, followed by 25 cycles at 95°C for 1 min, 58°C for 30 s, 72°C for 30 s, and 72°C for 7 min. A β-actin fragment (353-bp) was amplified and used as an internal control for the PCR. The amplified products were analyzed by

Construction of plasmids expressing short hairpin RNAs (shRNAs)
Three siRNA sequences targeting the human survivin, including SVV1, 5'-GGACCCCGCATCTTACA-3'; SVV2, 5'-GAAGCAGTTTGAAGAATT-3'; and SVV3, 5'-GAATTAACCCCCTGGAAT-3', were designed using the siRNA target finder tool (http://www.ambion.com/techlib/misc/siRNA_finder.html). The sense and antisense oligonucleotide chains expressing siRNA were synthesized (Sangon, Shanghai, China) by established methods with sequences as described previously [23]. Each siRNA gene fragment was flanked with a BamHI site at 5' end and a HindIII site at 3' end for cloning. Vectors expressing siRNA were designated as pSilencer3.1-SVV1, pSilencer3.1-SVV2, and pSilencer3.1-SVV3, respectively. The pSilencer3.1-H1neo-negative control vector was used as siRNA-negative control.

Transfection of PC-3 cells
Before transfection, cells (1 × 10⁵/well) were seeded in 6-well plates and grown to 90–95% of confluence. Prior to transfection, the medium was replaced by serum-free RPMI-1640 (2000 µl/well, without antibiotics). Serum-free RPMI-1640 (250 µl) containing 2 µg plasmid was mixed with 250 µl serum-free RPMI-1640 containing 5 µl Lipofectamine 2000™ reagents. The resultant mixture was kept at room temperature for 30 min, and then was added into the plates, 500 µl each well. The plates were incubated at 37°C for 4 h, and FBS (500 µl) was added into each well. Cells were then cultured for an additional 48 h before further analysis. The efficiency of transfection was monitored by the pEGFP-N2 vector (Promega) under IX71 inverted fluorescence microscope (Olympus, Japan).
1.5% agarose gel electrophoresis and semi-quantified using an image analysis system (FR200; Furi, Shanghai, China). The survivin mRNA levels from cells transfected with pSilencer3.1-SVV or with pSilencer3.1-NC empty vector were first normalized against β-actin. Then, the normalized survivin mRNA levels from pSilencer3.1-SVV-transfected cells were plotted as the percentage of expression relative to those from pSilencer3.1-NC-transfected cells.

**Western blot analysis**
The transfected PC-3 cells were washed with PBS and lysed at 4°C for 30 min in PBS containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 10% glycerin, and 1.0 mM phenylmethylsulfonylfluoride. Cell extracts were centrifuged at 12,000 g for 20 min at 4°C, and the supernatants (containing up to 300 µg of total proteins) were mixed with an equal volume of 2× SDS loading buffer. Samples were heated at 95°C for 5 min and were separated by SDS–15% PAGE and electrotransferred to nitrocellulose filters. The filters were blocked in PBS containing 5% skimmed milk and incubated overnight with the primary antibody specific for survivin, and then incubated with the secondary horseradish peroxidase-conjugated antibody.

Bound antibody was detected using the substrate solution of 0.1 ml/l diaminobenzidine-hydrogen peroxide. An anti-β-actin monoclonal antibody was used as an internal control. The results were semi-quantified using an image analysis system and the relative survivin protein levels were figured out with the similar methods used in calculation of the relative survivin mRNA levels.

**Flow cytometry (FCM)**
To examine cell apoptosis, transfected cells were fixed in 70% (v/v) ethanol, stained with an ANNEXIN V-FITC apoptosis assay kit (Becton–Dickinson, San Jose, USA) according to the instruction manual, and analyzed with a flow cytometer (FACSCalibur™, Becton–Dickinson). In some experiments, transfected cells were treated with 5.0 µg/ml platinol and were cultured for an additional 48 h prior to the flow cytometry (FCM) analysis.

For cell cycle analysis, transfected cells were stained with a CycleTEST™ PLUS DNA cell cycle assay kit (Becton–Dickinson) and then analyzed by FCM.

**MTT assay**
Transfected cells (2 × 10⁴) were seeded in 24-well plates and cultured in the presence of MTT [3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide]. At each time point, cells were harvested, lysed, and the absorbance (A) at 490 nm of the supernatants was measured using a Microplate Reader (Becton–Dickinson). Cell number was calculated by comparison with a standard curve of known number of cells. In some experiments, transfected cells were treated with platinol (5.0 µg/ml) before the analysis.

**In vivo inoculation of tumors**
Nude mice (eight for each group) were injected subcutaneously with 0.5 ml (2.0 × 10⁷/ml) PC-3 cells transfected with pSilencer3.1-SVV1, pSilencer3.1-SVV2, pSilencer3.1-SVV3, or pSilencer3.1-H1neo-negative control vectors. Tumor volumes were measured every day. Four weeks later, mice were sacrificed and tumor weights were measured.

**Statistical analysis**
Data were expressed as mean ± SD. Statistical significance was determined by the Student’s t-test and the SNK-q test. A value of P< 0.05 was considered as statistically significant.

**Results**

**Expression of survivin in prostate cancer cell lines**
We first examined the survivin expression in prostate cancer cell lines by immunohistochemistry. The results showed that the proportion of survivin-positive cells were 23% in PC3, 18% in DU145, 15% in LNCaP, 11% in 22RV1, and 12% in PC3M [Fig. 1(A)]. Immunoscors for PC3, PC3M, DU145, LNCaP, and 22RV1 were 49.42 ± 4.71, 28.45 ± 6.82, 34.27 ± 6.08, 34.09 ± 5.22, and 31.66 ± 7.13, respectively. The highest expression level of survivin was observed in the PC3 cells, compared with PC3M, DU145, LNCaP, and 22RV1. RT–PCR showed that the mRNA of survivin was expressed in five prostate cancer cell lines, which was confirmed by western blot, with the expression level higher in PC-3 and LNCaP cells [Fig. 1(B,C)]. Due to the highest expression of survivin in PC-3 cells among all tested prostate cancer cells, PC-3 was chosen as model cell in the following experiments.

**Silencing of survivin by RNAi inhibits cell proliferation and enhances chemosensitivity of prostate cancer cells**
The efficiency of transfection was ~75% as determined by pEGFP-N2 vectors [Fig. 2(A)]. To study the functions of survivin in prostate cancer cells, we employed the
RNAi strategy. PC-3 cells were transfected with siRNA-expressing vectors, and survivin mRNA and protein were measured by RT–PCR and western blot, respectively. The results indicated that pSilencer3.1-SVV2 and pSilencer3.1-SVV3 vectors were able to knockdown the expression of survivin [Fig. 2(B,C)]. The apoptosis index of PC-3 cells transfected with pSilencer3.1-SVV2 and pSilencer3.1-SVV3 increased greatly [Fig. 3(A)], whereas the proliferation of these cells decreased ~30%, as described previously [23]. In contrast with the control groups, the number of cells in the G1 phases of the siRNA group increased 10%, and cells in the G2 and S phases decreased ~5% [Fig. 3(B)]. Moreover, after treatment with platinol, the survival of the siRNA vector-transfected PC-3 cells decreased ~35–45% [Fig. 3(C)], whereas the apoptosis index increased ~29–42%, compared with control groups [Fig. 3(A)].

Knockdown of survivin in PC-3 cells attenuates its tumorigenicity in nude mice xenografts

To examine the effect of survivin on tumor formation by prostate cancer cells, we performed a tumorigenesis assay in nude mice. Nude mice were inoculated subcutaneously with PC-3 cells transfected with pSilencer3.1-SVV1, pSilencer3.1-SVV2, or pSilencer3.1-SVV3 vectors, respectively. Four weeks later, the mean volume and weight of tumors examined. The results showed that the volume and weight of the tumors derived from PC-3 cells

**Fig. 1 Expression of survivin in prostate cancer cell lines**

(A) The immunocytochemical staining of survivin in prostate cancer cell lines. (a) PC-3, (b) PC-3M, (c) DU145, (d) LNCaP, (e) 22RV1, and (f) negative control staining of survivin in PC-3M cells; magnitude, ×200. (B) RT–PCR of survivin mRNA in prostate cancer cell lines. (C) Western blot analysis of survivin in prostate cancer cell lines. β-Actin was used as control.
transfected with pSilencer3.1-SVV2 and pSilencer3.1-SVV3 vectors were 50–55% of those of the control groups (Fig. 4). These results indicate that the tumor formation and growth in nude mice xenografts were suppressed by silencing of survivin.

**Discussion**

Survivin is an anti-apoptotic protein, which is also involved in mitotic checkpoint control and apoptosis induced by growth factor in human cancer cells [24]. Survivin is overexpressed in a variety of human neoplasms. Currently, survivin expression has been used as a prognostic factor in several human neoplasms. High survivin expression by neoplasms correlates with more aggressive behavior, decreased responsiveness to chemotherapeutic agents, and shortened survival, when compared with cancers that are survivin-negative [25].

Recently, Zhang et al. [22] have shown that survivin mediates resistance to anti-androgen therapy with flutamide in prostate cancer cells. Specifically, these authors suggested that up-regulation of survivin via insulin-like
growth factor-1/AKT T-cell lymphoma (AKT) signaling during androgen blockade may be one of the mechanisms by which prostate cancer cells develop resistance to anti-androgens. In addition, survivin expression can be induced by heavy ion beams in some cancer cells, although the direct killing effect on cancerous cells by heavy ion radiation might be more significant than the anti-apoptosis effects of the survivin overexpression induced [26]. Overall, the results obtained in different studies indicate survivin to be a cellular factor potentially involved in the chemo-resistant and radiation-resistant phenotypes of human tumors cells and suggest that approaches designed to inhibit survivin expression may lead to human tumor sensitization to chemical and physical agents. McEleny et al. [27] found that the androgen-independent PC-3 and DU145 cells were highly resistant to apoptosis and expressed the highest levels of NAIP, XIAP, and survivin mRNA. Furthermore, Krajewska et al. [28] found that high expression of these IAP family proteins was evident in human prostate cancers and in prostate tissues from transgenic mice expressing SV40 large T antigen under the control of a probasin promoter. Positive survivin immunostaining was found in many invasive cancers but was not detected in normal prostate epithelium. Interestingly, positive immunostaining was found in occasional non-transformed prostatic epithelial cells of both transgenic mice and normal mice, associated with chromosomes or mitotic structures in cells apparently undergoing division.

In order to knockdown the overexpression of survivin in cancer cells, various strategies have been investigated. Multiple hammerhead ribozymes targeting the exposed regions of survivin mRNA synergistically produced the most potent anti-cancer effects and inhibited tumor growth in a hepatocellular carcinoma xenograft mouse model [29]. Uchida et al. [30] developed recombinant adenoviral vectors expressing siRNA against the survivin transcript and explored the impact of these vectors on three cancer cell lines. In all cell lines tested, knockdown of the survivin expression resulted in apoptotic cell death. Cancer cells infected with these vectors almost lost their tumorigenicity, following inoculation into nude mice. Intratumoral injection with the vectors significantly suppressed tumor growth in a mouse xenograft model. This novel strategy may be a promising tool for cancer gene therapy. Yang et al. [14] also showed that in the absence of survivin, certain euploid human cells undergo mis-segregation of chromosomes, abortive assembly of microtubules in late mitosis, failure of cytokinesis, and arrest of DNA synthesis. These results further indicate the significances of survivin in cancer.

In a word, survivin is highly expressed in the PC-3, LNCAP, and DU145 prostate cancer cells. The two pSilencer3.1-SVV2 and pSilencer3.1-SVV3 constructs could knockdown the expression of survivin in a similar manner [31]. PC-3 cells transfected with the siRNA expression vectors showed increased apoptosis, decreased proliferation, and increased sensitivity to the proapoptotic stimuli platinol. The tumorogenesis activity

Fig. 4 Survivin knockdown in PC-3 cells suppressed tumor formation in nude mice xenografts (A) The growth curve of tumor in nude mice bearing prostate cancer cell xenografts. (B) Tumors in nude mice bearing prostate cancer cell xenografts. (C) The tumor weight in nude mice bearing prostate cancer cell xenografts.
of these cells also decreased. These results are important in revealing the role of survivin in prostate cancer [32]. Due to the poor transfection efficiency, the mRNA and protein levels of survivin in prostate cancer cells could not be knocked down obviously enough in the experiments, further methods such as adenoviral or lentiviral delivery system can be used to overcome this disadvantage. A full understanding of effects of survivin in prostate cancer may lead to the development of new treatments for metastatic prostate cancer. Taken together, these findings indicate that survivin is significantly expressed in prostate cancer cells. Silencing of survivin expression by RNAi attenuated the malignant phenotypes of prostate cancer cells and may provide a novel approach for gene therapy of androgen-independent prostate cancer.

Funding

This work was supported by the grants from the National High Technology Research and Development Program of China (No. 2006AA02A245) and the National Natural Science Foundation of China (No. 30500433).

References

6 Li F and Ling X. Survivin study: an update of ‘what is the next wave’? Acta Biochim Biophys Sin (Shanghai) 2007, 39: 280–288.


