Therapeutic potential of siRNA-mediated combined knockdown of the IAP genes (Livin, XIAP, and Survivin) on human bladder cancer T24 cells

Deyong Yang1, Xishuang Song1*, Jianing Zhang2, Lin Ye1, Shujing Wang2, Xiangyu Che1, Jianbo Wang1, Zhiwei Zhang1, Lina Wang1, and Wei Shi2

1Department of Urology, First Affiliated Hospital of Dalian Medical University, Dalian 116011, China
2Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 116044, China

*Correspondence address. Tel: + 86-411-83635963-2102; Fax: + 86-411-83622844; E-mail: songxishuang@gmail.com

Livin, X-linked inhibitor of apoptosis (XIAP), and Survivin are three well-known inhibitors of apoptosis almost exclusively over-expressed in cancer cells and are considered potent targets for cancer treatment. In the present study, we found that Livin, XIAP, and Survivin were simultaneously expressed in bladder cancer cells. We speculated that Livin, XIAP, and Survivin might have synergistic effects on cell growth and apoptosis. Our results confirmed that combined knockdown of all these three genes can synergistically inhibit the proliferation and transformation ability of high-grade bladder cancer T24 cells and promote the cell apoptotic sensitivity to chemotherapy. Furthermore, combined knockdown of Livin, XIAP, and Survivin can markedly increase the abundance of active caspase-3, active caspase-7, active caspase-9, and cytosolic Smac. Our findings imply that combined silencing of Livin, XIAP, and Survivin may be a potent multi-targeted gene therapy for bladder cancer.

Keywords Livin; XIAP; Survivin; bladder cancer; multi-targeted therapy

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Introduction

Apoptosis deficiency is a hallmark of malignancy. Cancer cells often escape apoptosis through a number of mechanisms, among them over-expression of anti-apoptotic genes has been shown to play a critical role [1,2]. The inhibitors of apoptosis (IAPs) are a group of anti-apoptotic factors in the apoptotic pathway that render cancer cells insensitive to apoptotic stimulation [3,4]. Currently, eight mammalian IAPs have been identified in mammals, including X-linked inhibitor of apoptosis (XIAP), cellular IAP-1 (cIAP-1), cIAP-2, Livin, neuronal apoptosis inhibitory protein (NAIP), Survivin, and BRUCE [5–7]. Among them, Livin [8] and Survivin [9] have been proven to be extensively expressed in many types of cancers and not expressed or expressed at a substantially lower level in their normal tissue counterparts. XIAP [10] was considered as the most potent IAPs member in apoptosis inhibition and often over-expressed in cancers. Furthermore, the over-expression of Livin, XIAP, and Survivin in cancers is often associated with poor prognosis and resistance to radiotherapy and chemotherapy.

Due to the potential therapeutic value, several strategies have been used to overcome the apoptotic resistance of neoplastic populations [11]. One of the most promising strategies is the use of RNAi technology to suppress the expression of IAPs [12]. Previous studies have shown that knockdown of Livin, XIAP, or Survivin can, respectively, inhibit cell proliferation and enhance the apoptotic sensitivity of many types of cancer cells to chemotherapeutics [13–16]. However, Livin, XIAP, and Survivin, which are structurally similar, often simultaneously existed in cancer cells and functionally overlap in the apoptotic pathway. Therefore, suppressing all these three genes may remove the barricade in the cell apoptotic pathway more effectively than suppressing only a single gene.

Here, we used the high-grade bladder cancer cell line, T24, and RNAi to achieve individual or combined suppression of Livin, XIAP, and Survivin expression. The resulting effects on cell growth and apoptosis were analyzed to compare the effects of the knockdown of each individual gene with the effects of the combined knockdown of all these three genes.

Materials and Methods

Cell culture

Human bladder cancer cell lines T24, EJ, and BIU-87, a human uroepithelial SV-HUC-1 cell line, a human cervical cancer cell line, HeLa, and a human prostate cancer cell line, PC-3, were obtained from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All of
the cell lines were maintained in RPMI 1640 (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured under a humidified atmosphere of 5% CO₂.

**Reverse transcription–polymerase chain reaction analysis**

Total RNA was extracted from transfected cells using RNAiso reagent (Takara, Dalian, China) according to the manufacturer’s protocol, and 5 μg of total RNA was used to synthesize the first-strand of cDNA using an oligo(dT) primer and RT Superscript II, as recommended by the manufacturer (Takara). For semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR), 1 μl of the first-strand cDNA was used as a template for the PCR containing 3 μl of 10× PCR buffer, 1 μl of 10 mM dNTPs, 0.5 μl of each primer (20 pmol/μl), and 2 U of Taq DNA polymerase (Takara) in a final volume of 30 μl. Primers used are listed in Table 1. The following PCR protocol was used: 1 cycle at 95°C for 3 min; 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 5 min. PCR products were detected by electrophoresis on a 1% agarose gel and photographed under ultraviolet light. A DL2000 marker was used as the molecular weight standard.

### Table 1 Primers used in RT–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Livin</strong></td>
<td>Forward: 5'-GAGGCCAGTGGTTCCCTCCAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCAGGGCCAAAGACAGA-3'</td>
</tr>
<tr>
<td><strong>Survivin</strong></td>
<td>Forward: 5'-CTTTCTCAAGGACCAGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CTTACCAGCAGCACCC-3'</td>
</tr>
<tr>
<td><strong>XIAP</strong></td>
<td>Forward: 5'-GTGCCACGCAGTCTCAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCTCACTTCAAGG-3'</td>
</tr>
<tr>
<td><strong>β-actin</strong></td>
<td>Forward: 5'-AGCGAGCATCCCCCAAAGTT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGGCACGAAGGCTCATCATT-3'</td>
</tr>
</tbody>
</table>

**Western blot analysis**

Total proteins were extracted using a total protein extraction kit (KeyGEN, Nanjing, China) and mitochondrial proteins were fractionated using the mitochondria isolation kit (Pierce, Rockford, USA). Denatured proteins were mixed with 5× sodium dodecyl sulfate (SDS) sample buffer (0.5 M Tris–HCl, pH 6.8, 10% SDS, 5% glycerol, and 5% β-mercaptoethanol) and resolved by 12% SDS–polyacrylamide gel electrophoresis and then transferred onto PVDF membranes (Pall Corporation, San Diego, East Hills, USA). After blocking for 2 h with PBS containing 0.1% Tween 20 and 5% skimmed milk powder, the membranes were, respectively, incubated overnight in 5% skimmed milk powder buffer with rabbit polyclonal antibodies directed against human Livin, XIAP, Survivin, and Smac, or active caspase-3, active caspase-7, and active caspase-9 (Santa Cruz Biotech, Santa Cruz, USA) at 1:500 dilution. The membranes were washed three times with PBS containing 0.1% Tween 20 and followed by incubation with an HRP-conjugated anti-rabbit IgG as the secondary antibody (Santa Cruz Biotech) at 1:2000 dilution. Detection of GAPDH (Santa Cruz Biotech) at 1:500 dilution and porin was used as a loading control. All bands were detected using an ECL western blot kit (Amersham Biosciences, Buckinghamshire, UK).

**RNAi assay**

T24 cells were incubated in a six-well tissue culture dish without antibiotics for 24 h prior to transfection, resulting in 60–80% confluence. Negative control (NC) siRNA and specific Livin, Survivin, and XIAP siRNA transfection reagent complexes were mixed with Lipofectamine2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s recommendation and were added to the cells. After 6 h at 37°C, the medium was changed, and the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum for different times. Silencing of Livin, Survivin, and XIAP was determined by western blot assay. The siRNA sequences are shown in Table 2.

### Table 2 Information of siRNAs

<table>
<thead>
<tr>
<th>siRNA (FAM-labeled)</th>
<th>Target sequence</th>
<th>Accession no.</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Livin siRNA-1</td>
<td>5'-GGAAGAGACCTTTGTCACCA-3'</td>
<td>NM_022161</td>
<td>648–666</td>
</tr>
<tr>
<td>Livin siRNA-2</td>
<td>5'-GAGAGGTCAGTCGAAAG-3'</td>
<td>790–808</td>
<td></td>
</tr>
<tr>
<td>XIAP siRNA-1</td>
<td>5'-GAGGAAATTTGGAGATTAA-3'</td>
<td>NM_001167</td>
<td>3330–3348</td>
</tr>
<tr>
<td>XIAP siRNA-2</td>
<td>5'-GGCAUAGTTGGAGAAGGA-3'</td>
<td>6432–6450</td>
<td></td>
</tr>
<tr>
<td>Survivin siRNA-1</td>
<td>5'-GAAGAAAGAATATTGGAGA-3'</td>
<td>NM_022160</td>
<td>363–381</td>
</tr>
<tr>
<td>Survivin siRNA-2</td>
<td>5'-GAGACAGAATAAGAGTGA-3'</td>
<td>1144–1162</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>5'-TTCTCCGAAACGTGCAGT-3'</td>
<td>Non-targeting</td>
<td></td>
</tr>
</tbody>
</table>
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay

T24 cells, with and without transfection, were plated in 96-well plates at the indicated density in 100 μl of RPMI 1640 containing 10% fetal calf serum and cultured at 37°C for 3 days. Cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each group had three samples. The absorbance was read at 470 nm using a plate reader. The data points represent the mean ± SD of a quadruplicate determination from a representative experiment that was repeated at least three times.

Apoptosis assay

For detection of phosphatidylserine externalization, trypsinized cells were double stained with FITC-conjugated Annexin-V (25 μg/ml) and propidium iodide (PI) (50 μg/ml) apoptosis detection kit (Beckman–Coulter, Brea, USA). Cells (1 × 10^4) were collected on a FACScan flow cytometer equipped with a 488 nm argon laser and analyzed using the CellQuest software (Becton–Dickinson, Franklin Lakes, California, USA).

Soft agar colony formation assay

A soft agar formation assay was used to determine the effect of down-regulation of Livin, XIAP, and Survivin on the transformation capability of T24 cells. In brief, a bottom layer (0.6% low-melt agarose) was prepared with RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. A top layer (0.3% low-melt agarose) was prepared with the same RPMI 1640 medium as described above plus 5000 of the indicated cells. Plates were incubated at 37°C in 5% CO2 in a humidified incubator for about 2 weeks. The plates were then scanned and photographed, and the number of colonies was quantified by Quantity one v.4.0.3 software (Bio-Rad, Hercules, USA).

Statistical analysis

SPSS13.0 software was used to determine statistical significance. Each assay was performed at least three times. The data were expressed as the mean ± SD, and Student’s t-test was used to determine the significance of the differences in multiple comparisons. An asterisk stands for a P-value <0.05 and was considered statistically significant.

Results

Livin, XIAP, and Survivin expression in cancer cell lines

RT–PCR and western blot were used to determine the expression level of Livin, XIAP, and Survivin in three bladder cancer cell lines (T24, EJ, and BIU-87), as well as the human cervical cancer cell line (HeLa) and the human prostate cancer cell line (PC-3). As shown in Fig. 1(A), mRNA for Livin, XIAP, and Survivin was simultaneously expressed in each of these cell lines. Livin, XIAP, and Survivin proteins were detected by western blot in human bladder cancer T24 cells and human uroepithelial SV-HUC-1 cells. As shown in Fig. 1(B), the three IAPs were highly expressed in T24 cells, but not expressed, or expressed in extremely low level in SV-HUC-1 cells (P < 0.05).

siRNA-mediated knockdown of Livin, XIAP, and Survivin in T24 cells

To achieve efficient and specific silencing of the target genes, the two most effective siRNAs for Livin, XIAP, and Survivin were screened (data not shown) and mixed together equally as an siRNA pool. Western blot analysis was performed 72 h after the transfection to evaluate RNAi efficacy. As shown in Fig. 2(A), the level of Livin, XIAP, and Survivin proteins was significantly decreased by up to 70–80% by 30 nM of their specific siRNA. Furthermore, combined RNAi for all the three genes by 90 nM Livin–XIAP–Survivin (L–X–S) siRNA, containing 30 nM of each specific siRNA, did not decrease the knockdown efficiency of each individual target. Moreover, 30 nM L–X–S siRNA, containing 10 nM of each specific siRNA, also achieved an efficient triple knockdown [Fig. 2(B)].

IAP gene knockdown inhibited cell proliferation and clonogenic survival

Next, we determined the effect of siRNA of individual gene and combined knockdown of IAP genes on cell viability and proliferative ability using an MTT assay [Fig. 3(A)].
The optical density at the indicated time points of cells treated with Livin, XIAP, Survivin, and L–X–S siRNA was lower than that of the NC siRNA-treated cells. Moreover, the optical density of L–X–S siRNA-treated cells began to decline after 36 h. Similarly, the colony formation assay [Fig. 3(C)] showed that the total colony number of Livin, XIAP, Survivin, and L–X–S siRNA-treated cells was less than that of the NC siRNA-treated cells. The colony number of the L–X–S siRNA-treated cells was further decreased when compared with that of the cells transfected with each individual RNAi.

Colony formation assay was performed to further evaluate whether triple IAPs knockdown synergistically inhibits T24 cell transformation ability. To this end, the duration of transient RNAi effect caused by siRNA was evaluated by western blot in advance. The result showed that IAPs expression reverted to the original level on the eighth day after siRNAs transfection (data not shown), indicating that the siRNA could cause suppression for ~1 week in T24 cells. The result of colony formation assay was obtained on the 14th day. In spite of the recovery of IAPs expression 1 week after transfection, colony formation ability of T24 cells was significantly decreased in IAPs knockdown groups for 1 week in the absence of IAPs, compared with the control group. Furthermore, similar to the result of MTT assay, colony formation ability of T24 cells markedly decreased in the triple
IAPs knockdown group compared with the individual IAP knockdown groups.

Taken together, these results indicated that the combined knockdown of Livin, XIAP, and Survivin has a stronger effect on the inhibition of cell viability, proliferative capacity, and transformation ability in T24 bladder cancer cells compared with the effect of suppression of each individual gene.

IAP knockdown promotes apoptosis of T24 cells

To examine the contribution of IAPs knockdown to the apoptosis of T24 cells in vitro, Annexin-V and PI staining were used to detect apoptotic cells including early-stage apoptosis (AV+/PI−) and late-stage apoptosis (AV+/PI+). As shown in Fig. 4, individual IAP gene knockdown could increase spontaneous apoptosis to a small extent; however, triple IAP genes’ knockdown could trigger spontaneous apoptosis to a great extent, indicating that siRNA L–X–S itself had a direct therapeutic effect on bladder cancer T24 cells.

To investigate whether IAP genes’ knockdown enhances T24 cell sensitivity to chemotherapy, cell apoptosis was examined 48 h after transfection in the presence of a low concentration of mitomycin (5 µg/ml) for 24 h. As shown in Fig. 4, without IAP genes’ knockdown, mitomycin only led to 12.16 ± 2.08% apoptotic cells. However, individual knockdown of Livin, XIAP, and Survivin could promote apoptosis to more than 30%. Moreover, the triple IAP genes’ knockdown greatly increased apoptosis to nearly 80%, indicating that combined IAP genes’ knockdown had an obvious advantage over individual IAP gene knockdown in promoting T24 cell sensitivity to chemotherapy.

Triple IAP genes’ knockdown promotes cleavage of caspase-3, caspase-7, and caspase-9, and the expression of cytosolic Smac

The down-regulation of IAPs should lead to changes in downstream signaling pathways, including activation of caspase-3, caspase-7, and caspase-9. As shown in Fig. 5(A), in the presence of 5 µg/ml mitomycin for 24 h, the triple IAP genes knockdown significantly increased the presence of cleaved caspase-3, caspase-7, and caspase-9 compared with the knockdown of each individual gene. These data indicated that the combined knockdown of Livin, XIAP, and Survivin has a significant impact on apoptosis in bladder cancer T24 cells.

Due to the enhanced pro-apoptotic effect caused by the triple IAP genes knockdown, we further analyzed the level of the second mitochondria activator of caspase (Smac), a pro-apoptotic protein released from the mitochondria that can antagonize IAPs. As shown in Fig. 5(B), there were no significant differences in the mitochondrial Smac among IAP genes’ knockdown groups compared with the control group. Interestingly, the cytosolic Smac was mildly increased in siRNA Livin group and siRNA XIAP group and greatly increased in siRNA L–X–S group, but not in siRNA Survivin group when compared with the control group.

Discussion

A deficiency in apoptosis is a well-known hallmark of many cancers. Therapeutic strategies that target the apoptotic pathways tend to correct the apoptotic deficiency and have been widely studied to treat cancer [17]. Livin, XIAP, and Survivin are utilized by cancer cells to antagonize cell apoptosis. The expression of Livin, XIAP, and Survivin is often associated with poor prognosis and cancer cell resistance to radiotherapy and chemotherapy. Depletion of Livin, XIAP, and Survivin in cancer cells have all been considered as ideal targets for cancer therapy [12]. Until now, several studies have been carried out aiming at decreasing cancer cell growth and at the same time enhancing apoptotic sensitivity by down-regulating Livin, XIAP, or Survivin. However, Livin, XIAP, and Survivin are usually simultaneously expressed in cancer cells and have overlapping functions in cell growth and apoptosis.
According to the Science Signaling Pathway Database (http://stke.sciencemag.org/cm/), Livin, XIAP, and Survivin jointly inhibit both the extrinsic and intrinsic apoptotic pathways. Moreover, they functionally overlap in their interaction with caspase-3, caspase-7, caspase-9, and Smac (Fig. 6). Thus, the depletion of Livin, XIAP, and Survivin as a whole could more completely remove the barricade in the cell apoptotic pathway compared with withdrawing only one of them. Here, in an attempt to use RNAi to correct the apoptosis deficiency of tumor cells, we chose Livin, XIAP, and Survivin as a combinational target. We demonstrated that siRNA technology represents an extremely powerful tool to achieve specific and efficient multiple gene inhibition in bladder cancer T24 cells.

In this study, we observed that RNAi suppression of Livin, XIAP, and Survivin individually resulted in a mild decrease in cell growth and a modest increase in spontaneous cell apoptosis. In fact, several previous studies have indicated that the depletion of Livin, XIAP, or Survivin from cancers could enhance the radiosensitivity and chemosensitivity of cancer cells [13,15,18]. Here, we demonstrated that the combined suppression of Livin, XIAP, and Survivin has significant advantages over each individual knockdown in correcting the apoptosis...
deficiency of cancer cells. When triple knockdown was achieved, the growth of T24 cells was dramatically inhibited and the spontaneous cell apoptosis was strongly enhanced. Moreover, the sensitivity of T24 cells to chemotherapy drug, mitomycin, was greatly promoted by the triple IAPs suppression. Thus, in terms of a clinical perspective, combined suppression of Livin, XIAP, and Survivin by siRNA appears to be more promising.

The concentration of siRNA may also interfere with cell viability through off-target effects or interferon pathways [19,20]. Here, we used 30 nM Livin, XIAP, and Survivin siRNA to achieve a specific and efficient knockdown of the target genes separately. For combined RNAi, the L–X–S siRNA was used at both a 30 nM and a 90 nM concentration. In the present study, we demonstrated that although RNAi efficiency for an individual gene by 30 nM L–X–S siRNA was mildly weak compared with 30 nM of their specific siRNA, the triple knockdown still had a greater effect on cell growth and apoptosis than each individual IAP knockdown.

The effect of combined RNAi on cell growth and apoptosis observed in this study was consistent with previous experiments that showed that IAPs were inhibited by Smac, a pro-apoptotic protein released from the mitochondria [21]. The interaction of Smac with IAPs relieved the inhibitory effect of the IAPs on caspases. Specially, the RING domain of IAPs could degrade Smac by ubiquitination [22,23]. In this study, we observed that the cytosolic Smac was significantly increased in siRNA Livin, siRNA XIAP, and siRNA L–X–S groups, but not in siRNA Survivin group compared with siRNA NC group. This might be partially because that the knockdown of Livin and XIAP, which possess RING domain, decreased the degradation of Smac in cytosol. However, the exact mechanism requires further study.

In accordance with the effects of siRNA at the cellular level, the combined silencing of endogenous Livin, XIAP, and Survivin expression by siRNA led to an increase in the level of active caspase-3, caspase-7, and caspase-9. These results do not contradict with previous studies. In fact, several studies have already focused on the molecular mechanisms of IAPs and their upstream and downstream factors [22,24–26]. In addition to their role in apoptosis inhibition, IAPs have a number of other functions, including roles in cell cycle regulation, differentiation, immunity, ubiquitination, and targeting of proteins for degradation [6]. However, the exact mechanism by which these proteins inhibit apoptosis is complicated and still not totally understood.

In accordance with our present study, we believe that siRNA-based combined knockdown of Livin, XIAP, and Survivin may serve as a promising anticancer strategy to be used in clinics. This therapeutic approach is highly specific to the target genes and could avoid the complication of tumorigenesis and immune responses caused by vector-based RNAi strategies. Furthermore, chemically modified siRNAs are more resistant to be degraded in vivo and could be repeatedly used in systemic or local RNAi [27]. However, the specific and efficient delivery of therapeutic siRNAs into target organs remains a challenge. Notably, ultrasound-mediated siRNA transfection was reported to greatly promote the specificity and efficiency of transfection [28]. However, further in vivo studies are required to determine whether this technique can provide a new route for siRNA-mediated gene therapy.

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References


