RESEARCH ARTICLE

Impact of Co-transfection with *Livin* and *Survivin* shRNA Expression Vectors on Biological Behavior of HepG2 Cells

Wei Xu, Hong Chang*, Cheng-Kun Qin, Yun-Peng Zhai

Abstract

Objective: To construct short hairpin RNA (shRNA) eukaryotic expression vectors targeting Livin and Survivin genes, and to explore the impact of co-transfection of Livin and Survivin shRNA expression vectors on the biological behavior of HepG2 cells. Methods: shRNA eukaryotic expression vectors pSD11-Livin and pSD11-Survivin were designed and constructed then transfected into HepG2 cells separately or in combination. mRNA and protein expression in transfected cells was assessed by quantitative fluorescence PCR and Western blotting, respectively. Cell proliferation was measured by MTT assay and cell apoptosis by TUNEL assay. Results: The Livin and Survivin shRNA eukaryotic expression vectors were successfully constructed and transfected into HepG2 cells. The relative mRNA expression levels of Livin and Survivin in HepG2 cells co-transfected with pSD11-Livin and pSD11-Survivin were 0.12 ± 0.02 and 0.33 ±0.13, respectively, which was significantly lower than levels in cells transfected with either pSD11-Livin or pSD11-Survivin ($P<0.05$). The relative protein expression levels of Livin and Survivin in the co-transfected cells were also significantly decreased compared to single-transfection ($P<0.05$). The inhibition rate of cell growth in the co-transfection group was higher than that in the single-transfection groups at 48 h, 60 h, or 72 h after transfection ($P<0.01$). The apoptotic rate increased to the greatest extent in the co-transfection group relative to any other group ($P<0.05$). Conclusions: Co-transfection with pSD11-Livin and pSD11-Survivin was more efficient than transfection with either vector alone in reducing the mRNA and protein expression of Livin and Survivin genes in HepG2 cells. Co-transfection also inhibited the proliferation of transfected cells more than the other groups, and induced cellular apoptosis more effectively.

Keywords: Livin - Survivin - RNA interference - hepatocellular carcinoma

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Introduction

Liver cancer is one of the most common digestive malignancies. Conventional therapies such as surgical resection and chemotherapy are ineffective, and it is rare to achieve complete remission or cure. Application of RNA interference (RNAi) to reduce oncogene expression and thereby change the biology of tumor cells is one of the most important methods in tumor therapy (Sibley et al., 2010). Livin and Survivin are members of the Inhibitor of Apoptosis (IAP) family of proteins, and are highly expressed in hepatocellular carcinoma cells. These two genes exert their anti-apoptotic effects through the IAP repeat domains (baculovirus-IAP repeat, BIR). Knockdown of either Livin (Liu et al., 2010) or survin (Hung et al., 2012) can manifest therapeutic effects against liver cancer. It has also been shown that these two genes, together with XIAP, exert a synergistic effect that serves to inhibit cellular proliferation and to promote cell apoptosis in human bladder cancer cells (Yang et al., 2010) and human colon cancer cells (Oh, 2011; Carrasco et al., 2011). However, it remains unknown as to whether the combined inhibition of these two genes shows any synergistic effects on cellular proliferation and apoptosis in human liver cells. In the present study, we designed and constructed eukaryotic expression vectors that encode Livin and Survivin shRNAs. These vectors were transfected or co-transfected into hepatocellular carcinoma HepG2 cells, with the aim of exploring the effects of single and combinatorial gene silencing on the biological behavior of HepG2 cells.

Materials and Methods

Reagents

DMEM medium, fetal bovine serum (FBS), and OPTI-MEM medium were purchased from Gibco Corporation (Grand Island, NY), lipofectamineTM2000 transfection Reagent Kit was purchased from Invitrogen Corporation (Grand Island, NY); RNA extraction Kits, reverse transcription kits, and quantitative fluorescence PCR kits were purchased from Takara Company (Dalian, China); the protein extraction and quantification kit was purchased from BIOCOLOR Bioscience limited

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(Shanghai, China); anti-Livin, anti-Survivin, and anti-GAPDH (all mouse anti-human monoclonal antibodies) were purchased from Abcam Company (Hong Kong, China); horseradish peroxidase-labeled goat anti-mouse antibodies were purchased from Beijing Ding Guo Changsheng Company (Beijing, China); MTT from Sigma Corporation (Shanghai, China); and TUNEL Kits from Roche Company (Shanghai, China).

Cell culture

Human hepatoma HepG2 cells were obtained from the Shandong University Affiliated Provincial Hospital. HepG2 cells were cultured in DMEM supplemented with 10% FBS at 37°C, in an incubator with 5% CO_2. The cells adhered to the substratum were grown to confluence, and were digested with 0.25% trypsin for passage.

Construction of Livin and Survivin shRNA eukaryotic expression vectors

The full-length mRNA sequences of Livin (Gene ID: 79444) and Survivin (Gene ID: 332) genes were obtained from the NCBI database. The 19 nt siRNA target sequences were designed according to the literature (Hung et al., 2012) as follows: Livin: 5'-GGAAGAGACTTTGTCCACA-3' (648-666); Survivin: 5'-GGACCACCGCATCTCTACA-3' (166-184). As confirmed by Blast searching, the sequences showed low sequence identity with other human genes. BamH I and Hind III restriction sites were added to the 5' and 3' ends, respectively. Between the strand and antisense strands was a 9 nt ring sequence (TTCAAGAGA). The two sequences were separately cloned into the plasmid pSD11 by ligating the BamHI/HindIII-digested shRNA fragment to the vector after digestion using the same restriction endonucleases. Recombinant plasmids are shown in Figure 1, and were designated as pSD11-Livin and pSD11-Survivin, respectively. Simultaneously, a negative control plasmid pSD11-NC was constructed that encodes a shRNA of unrelated sequence. The construction of eukaryotic expression plasmids and PCR identification were conducted by the Shanghai Giquet Biotechnology Companies (PRC). The vectors were sequenced by the Shanghai Bio-Engineering LLC.

Transfection of cells

One day prior to transfection, the trypsin-digested HepG2 cells were inoculated onto a six-well plate, at a density of 3x105 cells per well. The cells were cultured for 24 h in DMEM with 10% FBS until about 80% confluency, as observed under a compound light microscope. Transfections were conducted according to the LipofectamineTM 2000 Transfection Reagent Kit instructions. The cells were divided into five groups: transfection with pSD11-Livin; transfection with pSD11-Survivin; transfection with both pSD11-Livin and pSD11-Survivin; transfection with pSD11-NC; and controls with no transfection.

mRNA expression as assessed by quantitative fluorescence PCR

Forty-eight hours after transfection, the total RNA was extracted from the transfected cells. Reverse transcription was performed according to the RT Kit instructions; at 37°C for 15 min, 85°C for 5 sec, and then the mix was left standing at 4°C. The primers were designed using Primer 5.0 software, following the guidelines for primer design in quantitative fluorescence PCR. The 5' primer of the Livin gene was 5'-GGTGAGGTGCTTCTTCTGCTATGG-3', and the 3' primer was 5'-GGCTGCGTCTTCCGGTTCTT-3'. The expected size of the amplified product was 199 bp. The 5' primer of Survivin was 5'-CTGTGCTCTTGTTTTGT CTTGA-3', and the 3' primer was 5'-CTTCTTCCTCCCTC ACTTCTCAC-3'. The expected size of the amplified segment was 166 bp; The 5' primer for GAPDH was 5'-AGAAGGCTGGGGCTCATTTG-3'; the 3' primer was 5'-AGGGGCCATCCACAGTCTTC-3', and the size of the amplified fragment was 258 bp. The primers were synthesized by Shanghai Bio-Engineering Biotechnology Corporation (PRC). Real-time quantitative PCR was performed using the SYBRgreen Chimeric fluorescence method in a Light-Cycler 480 (Roche, Switzerland). The PCR cycle parameters were: predegeneration at 95°C for 30 sec; denaturation at 95°C for 5 sec, and anealing at 60°C for 20 sec; for a total of 40 cycles. The melting curve analysis was performed at 65°C for 15 sec. A blank control without cDNA was used for all experiments.

The melting curve of the PCR product peaked at 89.8°C for Livin, 86.1°C for Survivin, and 88°C for GAPDH and all were observed as single peaks, ruling out non-specific amplification. The melting curve for the negative control without cDNA template exhibited a peak that differed from the peaks in the experimental groups (ruling out false-positive results). Relative gene expression was quantified using the 2-ΔΔCT method with a reference gene as internal standard.

Western blot

Survivin and Livin protein expression was assessed by Western blot. Forty-eight hours after transfection, the total protein was extracted from the cells in each group. After 10% SDS gel electrophoresis, the protein was transferred to PVDF membranes, followed by blocking with TBST buffer containing 10% fat-free milk powder at 37°C for 60 min. The first antibody (anti-Livin, anti-Survivin or anti-GAPDH), was then added, and the membrane was incubated at 4°C overnight. After washing with TBST buffer, secondary antibody was added to the membrane, and the membrane was shaken at room temperature for 60 min. After one additional washing, ECL solution was added and the membrane was incubated for 5 min. The membrane was then developed by a gel-imaging device developer, and imaged with the Alpha Electrophoresis Imager 2,200 Analyzer (Willoughby, OH).

Evaluation of cell growth inhibition rate (IR) by MTT assay

One day prior to transfection, HepG2 cells were seeded into 96-well plates, at a density of approximately 5x103 cells/well. The cells were divided into five groups as above, with five wells in each group. The transfection procedure was according to the manual instructions in the

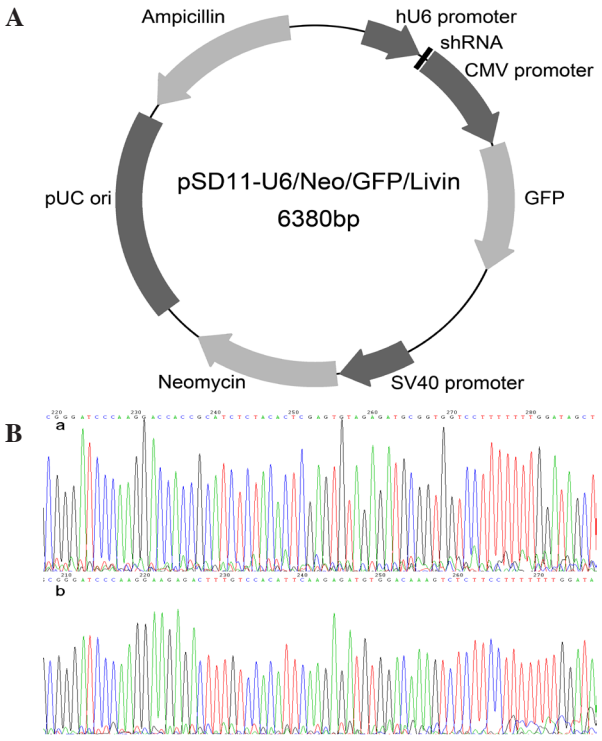

Figure 1. Construction of shRNA Expression Vectors. (A) Structure of recombinant plasmid pSD11 (6380 bp). Vector information (in bp): hU6 promoter: 289- 616; Polylinker: 617- 640; CMV promoter: 671- 1259; GFP: 1283- 2002; SV40 promoter: 2672- 3049; Neomycin: 3077- 3871; pUC ori: 4108- 5276; Ampicillin: 6233- 5373; (B) Sequencing of recombinant plasmids (a: Livin; b: Survivin)

LipofectamineTM2000 transfection Reagent Kit. After transfection, cells were cultured for 48 h, 60 h, or 72 h, respectively. The media were removed and 30 μl of MTT (5 mg/ml) was added to each well. The plate was incubated at 37°C, in an incubator with 5% CO_2 in compressed air and high humidity for 4 h; followed by the addition of 150 μl DMSO to each well. After shaking for 10 min, the plate was analyzed at an absorbance of 490 nm (OD value). The cell growth inhibition rate (IR) was calculated using the formula: IR= (1-OD value of the experimental group/OD of control group) x100%.

Detection of apoptosis by TUNEL assay

Forty eight hours after transfection, the cells were smeared on slides and fixed with 4% paraformaldehyde at room temperature for 30 min. In accordance with the TUNEL Kit instructions, the slides were thrice rinsed with PBS for 5 min each between each step. A solution without deoxyribonucleic acid transferase was used as the negative control for each group. The TUNEL-stained slides were observed under a light microscope. Apoptotic cells were identified and counted from five high-magnification views that were chosen randomly. The rate of apoptosis was calculated by the following formula: (Number of apoptotic cells counted in the five views /Total number of cells counted in the five views) × 100%.

Statistical analysis

SPSS 15.0 software was used for statistical analysis. Data were expressed as mean ± SEM. The statistical significance was evaluated by one-way ANOVA with Tukey's test. P values less than 0.05 were considered statistically significant.

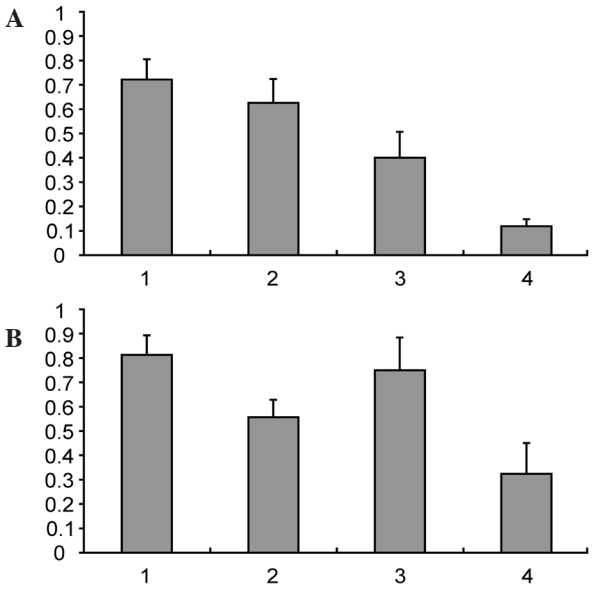

Figure 2. Livin and Survivin mRNA Expression in HepG2 Cells after Transfection or Co-transfection with Livin and Survivin shRNA Expression Vectors. The mRNA expression of Livin (A) and Survivin (B) was measured by real-time PCR. 1: plasmid control; 2: pSD11-Survivin transfection; 3: pSD11-Livin transfection; 4: Co-transfection with pSD11-Survivin and pSD11-Livin. *P<0.05 compared with the plasmid control; $^{\triangle}P$<0.05 compared with Survivin or Livin shRNA vector transfection (pSD11-Survivin or pSD11-Livin transfection alone)

Results

Knockdown of Livin and Survivin mRNA expression in HepG2 cells after co-transfection with Livin and Survivin shRNA expression vectors

The constructed plasmids, pSD11-Livin and pSD11-Survivin, were identified by PCR and DNA sequencing (Figure 1A, B). After transfection or co-transfection of HepG2 cells with these plasmids, the mRNA expression levels of Livin and Survivin in transfected cells were measured by real-time quantitative fluorescence PCR. Forty-eight h after transfection, the relative expression levels of Livin mRNA in HepG2 cells were 0.67 ± 0.18 for the plasmid control and 0.60 ± 0.14 (pSD11-Survivin transfection), 0.39 ± 0.12 (pSD11-Livin transfection), and 0.12 ± 0.02 (pSD11-Livin and pSD11-Survivin co-transfection). The relative expression levels of Survivin mRNA were 0.80 ± 0.09 for the controls and 0.74 ± 0.14 (pSD11-Survivin transfection), 0.55 ± 0.08 (pSD11-Livin) and 0.33 ± 0.13 (co-transfection with pSD11-Livin and pSD11-Survivin). Compared with single-gene transfection (pSD11-Livin or pSD11-Survivin), co-transfection with both pSD11-Livin and pSD11-Survivin was more efficient in reducing the expression of either Livin or Survivin gene (p<0.05) (Figure 2A, B). These results suggest that transfection of HepG2 cells with pSD11-Livin or pSD1-Survivin consistently silences the corresponding mRNA expression, and co-transfection with two plasmids

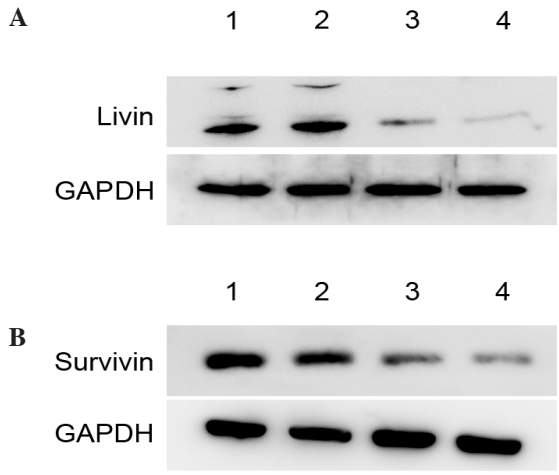

Figure 3. Livin and Survivin Protein Expression in HepG2 Cells after Transfection or Co-transfection with Livin and Survivin shRNA Expression Vectors. The protein expression of Livin (A) and Survivin (B) was examined by Western blot. 1: blank; 2: plasmid control; 3: pSD11-Livin transfection (A) or pSD11-Survivin transfection (B); 4: Co-transfection with pSD11-Survivin and pSD11-Livin

produces higher efficacy than transfection with either plasmid alone.

Knockdown of Livin and Survivin protein expression in HepG2 cells after co-transfection with Livin and Survivin shRNA expression vectors

In order to evaluate gene silencing at the protein level, we performed Western blotting to measure protein expression in transfected cells. The results showed that, after transfection for 48 h, the relative protein expression levels of Livin in transfected HepG2 cells were 0.78 ± 0.04 (blank controls), 0.79 ± 0.07 (plasmid controls), 0.41 ± 0.10 (co-transfection with pSD11-Livin and pSD11-Survivin), and 0.57 ± 0.03 (pSD11-Livin). The protein expression of Livin in Livin-transfected or co-transfected HepG2 cells was significantly lower than that in plasmid controls or blank controls (P<0.05). Furthermore, Livin protein expression in co-transfected cells was lower than that in Livin-transfected cells (P<0.05) (Figure 3A).

Likewise, the relative protein expression levels of Survivin in transfected HepG2 cells were 0.92 ± 0.07 (blank controls), 0.90 ± 0.06 (plasmid controls), 0.47 ± 0.05 (co-transfection with pSD11-Livin and pSD11-Survivin), and 0.54 ± 0.07 (pSD11-Survivin transfection). The Survivin protein expression in co-transfected or Survivin-transfected HepG2 cells was significantly lower than that in cells transfected with blanks or plasmid controls (P<0.05). The protein expression of Survivin was further decreased in co-transfected HepG2 cells when compared to that in pSD11-Survivin-transfected cells (P<0.05) (Figure 3B).

Transfection with Livin and/or Survivin shRNA vectors inhibits the growth of HepG2 cells

The MTT colorimetric assay showed that transfection of Livin or Survivin shRNA resulted in different degrees of growth inhibition in transfected HepG2 cells. The growth rate of HepG2 cells co-transfected with pSD11-Livin and pSD11-Survivin showed a higher inhibition rate compared with either the pSD11-Livin or pSD11-Survivin transfection group. (P<0.01) (Table 1).

Table 1. Growth Inhibition rate (%) in HepG2 Cells after Transfection

	48 h	60 h	72 h
Plasmid control	10.86±0.18	7.57±0.47	6.08±0.36
pSD11-Livin	19.60±0.48*	14.28±0.78*	9.70±1.02*
pSD11-Survivin	18.97±0.64*	13.84±0.69*	9.43±0.83*
Cotransfection	28.05±0.79*#	21.44±0.13*#	15.02±1.44*#

*P<0.01 vs. plasmid control. #P<0.01 vs. Livin or Survivin group; pSD11-Livin: Livin shRNA expression vector; pSD11-Survivin: Survivin shRNA expression vector

Transfection with Livin and/or Survivin shRNA vectors promotes apoptosis of HepG2 cells

Cellular apoptosis was detected by TUNEL assay. The apoptotic cells manifested brown staining in their nuclei, visible karyopyknosis, chromatin margination, condensate manifolds and scattered apoptotic bodies. Forty-eight h after transfection, the rate of apoptosis was (51.17 ± 4.86)% in co-transfected HepG2 cells, significantly higher than that in pSD11-Livin-transfected cells (40.78 ± 4.32)% or pSD11-Survivin-transfected cells (36.70 ± 3.97)% (P <0.05).

Discussion

Tumor occurrence and progression are the result of a combination of many factors, among which are the behavioral changes in cell biology. A number of investigators have proposed a 'multi-gene synergistic hypothesis', in which alterations in two or more genes may synergize to regulate behavioral changes in cells (Song et al., 2008). RNAi is a phenomenon of posttranscriptional gene silencing, in which double-stranded RNA specifically induces the degradation of homologous mRNAs. Currently, there are multiple methods to induce RNA interference in mammals (Apasani, 2007), one of which is to design a shRNA expression vector based on the siRNA of a target gene, and to introduce the vector into mammalian cells. A relatively stable and specific RNAi effect can then be produced, which avoids interferon-γ responses caused by direct introduction of siRNA into cells (Brummelkamp et al., 2002). This approach facilitates studies on combinatorial inhibition of multiple genes and their effects on biological behavior of tumor cells.

Unlike Bcl-2, the IAP is a class of anti-apoptotic proteins that play an inhibitory role in apoptosis within cells. Livin and Survivin are two members of the IAP family (Kenneth et al., 2012). The Livin gene is located on human chromosome 20Q13.3, and is approximately 46 kb in length. After posttranscriptional processing, two mature mRNA subtypes, Liven-α and Livin-β, are generated. Many studies have shown that Livin is highly expressed in some tumor tissues and cell lines. The shRNA expression vectors have been constructed and used to stably silence the Livin gene in the Lovo colon cancer cell line (Wang et al., 2008) and the SKOV3 ovarian cancer cell line (Liu et al., 2012), promoting apoptosis of

these cells. The Survivin gene is located on chromosome 17q25, and is 14.7 kb in length. It has been shown that upregulation of Survivin is associated with a decrease in tumor apoptosis index, increased metastasis rate, shorter survival times, poor prognosis, and increased recurrence rate (Augello et al., 2009). It has been reported that transfection of Panc-1 pancreatic cancer cell lines (Song et al., 2013) and the ovarian cancer cell line SKOV3 (Xing et al., 2012) with shRNA expression vectors effectively silenced the expression of Survivin gene in these cells and induced tumor cell apoptosis. Our previous studies also demonstrated that Livin and Survivin genes were highly expressed in a hepatocellular carcinoma cell line, which is closely related to the development of liver cancer in situ (Chang et al., 2008).

Currently, most studies report suppression of only one gene of the IAP family. We believe that inhibition of one gene of the IAP family causes changes in the expression of other genes in the family. Further, Livin and Survivin are members of the IAP family of relatively low molecular weights. They are structurally similar, and may exert synergistic effects in inhibiting apoptosis. This has been demonstrated in the report that siRNA knockdown of Livin, Survin and XIAP (another member of IAP family) can synergistically promote apoptosis of human bladder cancer cells, as each of these molecules inhibits apoptotic pathways, and their functions are overlapping (Yang et al., 2010).

In the present study, we selected one target-template sequence from Livin or Survivin mRNA sequences to construct shRNA eukaryotic expression vectors. The validity of the two selected target sequences was confirmed by preliminary experiments. By using quantitative fluorescence PCR and Western blotting, we measured the relative expression of mRNA and protein of the two genes, and demonstrated that the mRNA and protein expressions were significantly reduced in co-transfected HepG2 cells. Compared with transfection using a single shRNA vector, the mRNA and protein expressions in co-transfected HepG2 cells were decreased even further. The results of the MTT assay showed that the inhibition rate of HepG2 cells after co-transfection was significantly increased compared to that with single transfection. The TUNEL results also confirmed that apoptosis of HepG2 cells co-transfected with two shRNA vectors was increased to a greater extent compared to transfection with a single shRNA vector. Our results showed that, compared with the single gene suppression, co-transfection with pSD11-Livin and pSD11-Survivin was more effective in reducing Livin and Survivin gene expression in HepG2 cells and promoted apoptosis. In addition, quantitative PCR results showed that the inhibition of a single gene of the IAP family did not cause changes in the expression of other genes, indicating that Livin and Survivin gene expression are independent of each other; this is in contradistinction to our original hypothesis. However, co-transfection of the two genes resulted in a significantly higher cell growth inhibition rate and a higher apoptotic rate than with single transfection, suggesting that Livin and Survivin genes possess synergistic effects in inhibiting apoptosis.

Studies have shown that IAP affects the biological behavior of tumor cells by altering apoptotic pathways (Lau et al., 2012). The shared BIR domain in IAP family proteins interacts with caspases, the core molecules of apoptosis, to affect their activity so as to inhibit apoptosis Crnkovie-Mertens I et al. found that downregulation of Livin expression in Hela cells by RNAi resulted in increased caspase-3 expression (Crnkovie-Mertens et al., 2003); and Huh reported that Survivin inhibited apoptosis by inhibiting the activity of caspase-7 and caspase-3 (Liam et al., 2011). We confirmed in this study that co-transfection with Survivin and Livin shRHA expression vectors was more effective in inhibiting tumor gene expression in liver cancer cells and in inducing apoptosis than transfection with either Survivin or Livin shRNA expression vector alone. However, elucidation of the mechanism(s) underlying the interaction between these two genes--as well as their binding sites--is still to be investigated at the molecular level, and this would provide experimental evidence for a synergy between the two genes.

In summary, our results suggest that shRNA-based combined knockdown of both Livin and Survivin genes represents a promising strategy for the treatment of liver cancer. The synergy of the combined knockdown of these two genes suggests that targeting multiple genes by this strategy should produce robust efficacy against a variety of tumors.

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