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ORIGINAL ARTICLE

Effects of shRNA-silenced livin and survivin on lung cancer cell proliferation and apoptosis

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Summary

Purpose: *To evaluate the effects of short hairpin RNA (shRNA)-mediated silencing of livin and survivin on the proliferation and apoptosis of A549 lung cancer cells.*

Methods: *We designed and constructed the eukaryotic expression vectors pSilencer-livin and pSilencer-survivin which contain the Livin and Survivin genes, respectively, and transfected them into liposome-combined lung cancer A549 cells. The cells were then divided into the blank control, plasmid control, Livin, Survivin, and co-transfected groups. Real-time quantitative PCR (qRT-PCR) and Western blot assay were used to determine the mRNA and protein expression levels of Livin and Survivin. The MTT assay was used to evaluate the changes in cell proliferation. The TUNEL assay was used to evaluate the apoptotic rate.*

Results: *The shRNA eukaryotic expression vectors of Livin and Survivin were successfully constructed. The mRNA and protein expression of Livin and Survivin were significantly lower in the co-transfected group than in the control groups ($p<0.05$). At 48, 60, and 72 hrs after transfection, the cell growth inhibition rate was significantly higher in the co-transfected group than in the single-transfected group ($p<0.05$). At 48 hrs after transfection, the apoptotic rate significantly increased ($p<0.05$).*

Conclusion: *Co-silencing of Livin and Survivin can effectively inhibit the cell proliferation and apoptosis of lung cancer cells.*

Key words: *Livin, lung cancer A549 cell, short hairpin RNA (shRNA), Survivin*

Introduction

Lung cancer is a common malignant tumor representing a great health problem. This disease ranks first among males and third among females and accounts for 33.3% of the overall cancer mortality [1-3]. Although progress has been made in lung cancer surgery, chemotherapy, and radiotherapy, the 5-year survival rate of this disease remains less than 10% and has not shown significant improvement [4,5]. Tumorigenesis and development are caused by several factors, including cell biological behavior changes, which are major contributors to tumor evolution and progression. In tumor development, two or more changes in gene expression often synergize in the behavioral changes in cell biology [6-8]. RNA interference (RNAi), a vital research tool in tumor therapy, is used to reduce tumor gene expression in order to modify the biological activities of tumor cells [9-11]. Livin and Survivin are members of the inhibitor of apoptosis protein (IAP) family, which is highly expressed in lung cancer cells and exerts antiapoptotic effects through the IAP repeat domain (baculorius-IAP repeat). These two genes may exhibit synergistic effects on the occurrence, development, and metastasis of lung cancer [12-14].

In this study, we designed and constructed shRNA eukaryotic expression vectors of Livin and Survivin to silence the expression of Livin and Survivin alone or combined with the RNAi method. The effects of the shRNA eukaryotic expression vectors on the proliferation and apoptosis of A549 lung tumor cells were then investigated.

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Methods

Cell culture

A549 lung cancer cell line (Chinese Academy of Sciences Shanghai Institute of Cell Biology) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C in 5% CO_2 atmosphere. The adherent cells were passaged after digestion with 0.25% trypsin. Cells at the logarithmic growth phase were used in the experiments.

Vector construction

19 nt siRNA target sequences were selected after searching the full-length mRNA sequence of Livin and Survivin in the NCBI database and on the basis of the shRNA design principles and articles. The sequences are Livin: 5'-GGAAGAGACTTTGTCCACA-3' (648 bp to 666 bp) and Survivin: 5'-GGACCACCGCATCTCTACA-3' (166 bp to 184 bp). The blast analysis ensured that the selected sequences were not homologous with other human gene sequences. BamHI and HindIII restriction sites were designed at both ends of the sequence, and a 9 nt loop sequence (TTCAAGAGA) was designed between the sense and antisense strands. Both sequences were applied to the plasmid vector pSilencer4.1. ShRNA, which was synthesized with restriction sites at both ends, was directly attached to the digested carrier after annealing. After an overnight reaction of the target fragment to the plasmid vector (3:1) at 16 °C, the harvested product was stored at -20 °C. The ligation product (10 μL) was added to a 100 μL of precooled (0 °C) competent cells for transformation. After transforming, single white colonies were picked and cultured overnight, and the cells were then collected and sequenced.

Cell transfection and grouping

A day before transfection, the cells were digested with trypsin and were then plated in 6-well plates containing DMEM supplemented with 10% FBS at a density of 3×10^5 cells/well. The cells were then cultured for 24 hrs. When cell fusion was approximately 80% (as determined by microscopy), the transfection was performed in accordance with the instructions of the Lipofectamine TM 2000 transfection kit. The cells were subsequently divided into the Livin group (transfected with pSilencer-Livin), Survivin group (transfected with pSilencer-Survivin), co-transfected group (simultaneously transfected with both pSilencer-Livin and pSilencer-Survivin), plasmid control group (transfected with pSilencer 4.1), and blank control group (no transfection).

Real- time quantitative PCR

At 48 hrs after transfection, total RNA was extracted from each group and reverse-transcripted into cDNA in accordance with the reverse transcription kit instructions. The reaction was performed under the following conditions: 37°C for 15 min, 85°C for 5 sec, and then kept at 4 °C. The primers were synthesized by Shanghai Biological Engineering Co., Ltd. using the following sequences: Livin P1 5'-GGTGAGGTGCTTCTT CTGCTATGG-3' and P2 5'-GGCTGCGTCTTCCGGTTCTT-3', amplified fragment length was 199 bp; Survivin sense 5'-CTGTGCTCTTGTTTTGTCTTGA-3' and antisense 5'-CTTCTTCCTCCCTCACTTCTCAC-3', amplified fragment length was 166 bp; and GAPDH sense 5'-AGAAGGCTGGGGCTCATTTG-3' and antisense 5'-AGGGGCCATCCACAGTCTTC-3', amplified fragment length was 258 bp. SYBR green I real-time fluorescence quantitative PCR was performed on Roche's Light-Cycler 480 under the following reaction conditions: denaturation at 95 °C for 30 sec, 95 °C for 5 sec, and 60 °C for 20 sec for 40 cycles. Melting curve analysis was conducted at 65 °C for 15 sec. A blank control group with no cDNA was established for each group. Relative quantification was performed based on an internal reference standard gene. The amount of relative gene expression was $2^{-\Delta\Delta CT}$.

MTT

One day before transfection, the cells were plated in 96-well plates at a density of 5×10^3 cells/well. The cells were divided into the aforementioned groups with 5 wells in each group. At 48, 60, and 72 hrs after transfection, 30 μL of MTT (5 mg/mL) were added to each well after discarding the medium. The cells were then incubated for 4 hrs at 37 °C in 5% CO_2 atmosphere. Afterward, 150 μL of DMSO were added into each well, and the cells were shaken for 10 min before the optical densities (ODs) were determined at 490 nm. The cell growth inhibition rate (IR) was calculated using the following equation: IR = (1 - OD value of each experimental group/OD value of the blank control group) × 100%.

Cell apoptosis

At 48 hrs after transfection, the samples were prepared as previously described and then fixed using 4% paraformaldehyde for 4 hrs at room temperature. Groups were then established as previously mentioned. The experiment was performed in accordance with the TUNEL kit instructions. The samples were rinsed once with PBS for 5 min between each step. A negative control was established for each group by replacing the TUNEL reaction buffer with a marker fluid without a terminal deoxynucleic acid transferase. TUNEL staining was then performed on 5 randomly selected horizons from each group under optical microscope. The rate of apoptosis was then calculated.

Statistics

Statistical analysis was performed using the SPSS 15.0 statistical software (SPSS Inc, Chicago, IL). Stu-

dent's *t*-test data were presented as mean±standard deviation (x±s). One-way ANOVA was used to detect significant differences among treatment groups and statistical significance was set at $p<0.05$.

Results

Vector construction

The appearance of 367 and 322 bp bands during the agarose gel electrophoresis of BamHI and Hind III double-digested samples confirmed the construction of the designed vectors (Figure 1). Sequencing confirmed that the recombinant plasmid pSilencer-Livin and pSilencer-Survivin coding sequences were exactly as designed. Restriction enzyme digestion and DNA sequencing results indicated the correct insertion of the shRNA sequences into the plasmid pSilencer as well as the successful construction of the vectors.

Real-time quantitative PCR

The melting curves of the Livin, Survivin, and GAPDH PCR products peaked at 89.8, 86.1, and 88 °C, respectively. Only a single peak was common in all of the curves, and nonspecific amplification was excluded. At 48 hrs after transfection, the relative Livin mRNA expression levels were 0.539±0.127 (plasmid group), 0.501±0.115 (Survivin group), 0.393±0.123 (Livin group), and 0.121±0.012 (co-transfected group). The relative Survivin mRNA expression levels were 0.823±0.067 (plasmid group), 0.601±0.125 (Survivin group), 0.593±0.126 (Livin group), and 0.321±0.132 (co-transfected group). The decrease in the relative amount of mRNA was more significant in the co-transfected group than in the single-transfected group ($p<0.05$, Figure 2). These results demonstrated that A549 cell transfection by pSilencer–Livin and pSilencer–Survivin can specifically silence the expression of the corresponding mRNA. Moreover, co-transfection yielded better results than single transfection.

Western blot assay

Western blot assay was performed to analyze the Livin and Survivin expression. The results showed that 48 hrs after transfection, the relative amounts of Livin in the A549 cells were significantly lower in the co-transfection and Livin groups than in the plasmid and blank control groups ($p<0.05$). Furthermore, the relative amount of Livin in the co-transfected group was significantly lower than that in the Livin group ($p<0.05$, Figure 3). The

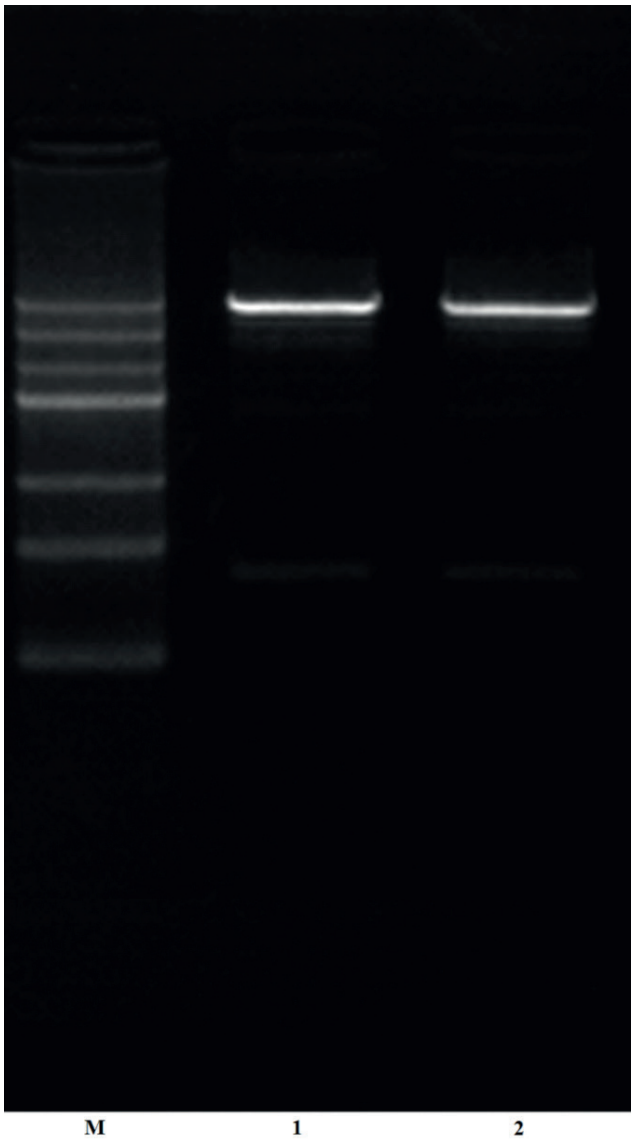

Figure 1. Electrophoresis of enzyme digested pSilencer-Livin and pSilencer-Survivin vector. M: DNA marker; 1: pSilencer-Livin; 2: pSilencer-Survivin.

Survivin expression was similar to that of Livin, but the relative amounts of Livin in the A549 cells were significantly lower in the co-transfection and Livin groups than in the plasmid and blank control groups ($p<0.05$). The expression in the co-transfected group was also significantly lower than that in the Livin group ($p < 0.05$, Figure 4).

MTT

MTT assay results indicated that the cells in each experimental group showed different degrees of growth inhibition. The rate of growth inhibition was significantly higher in the co-transfected group than in the Livin and Survivin groups ($p<0.05$, Table 1).

TUNEL assay

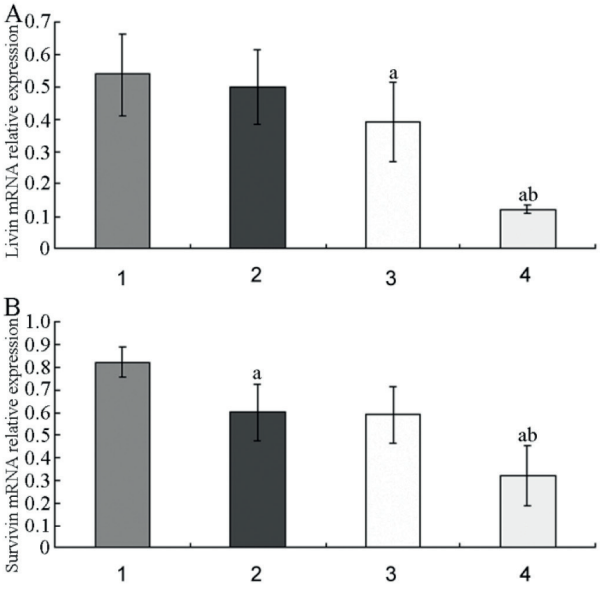

Figure 2. Relative expression of Livin and Survivin mRNA in A549 lung cancer cells from each group detected by real-time quantitative PCR. **a**:Livin mRNA; **b**:Survivin mRNA. 1:Plasmid control group; 2:Survivin group; 3:Livin group; 4:Co-transfected group. [a]p<0.05 vs the Livin group; [ab]p<0.05 vs the Survivin group.

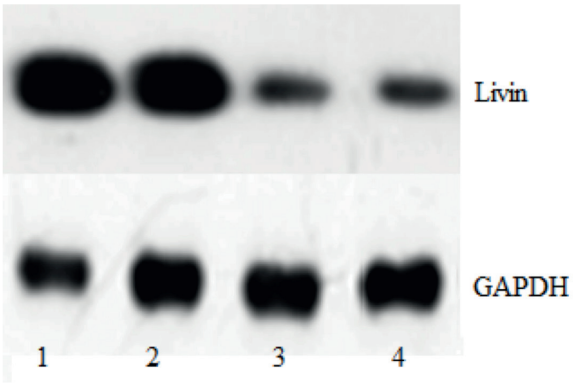

Figure 3. The relative expression of Livin protein in A549 lung cancer cells from each group detected by Western blot. 1:Blank control group; 2:Plasmid control group; 3:Livin group; 4:Co-transfected group.

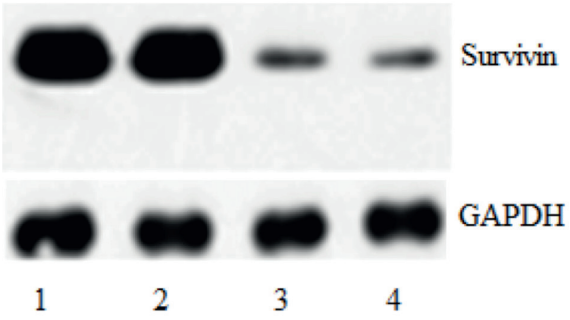

Figure 4. Analysis of the relative expression of Survivin protein in A549 lung cancer cells from each group detected by Western blot. 1:Blank control group; 2:Plasmid control group; 3:Survivin group; 4:Co-transfected group.

The nuclei of apoptotic cells were stained brown, and karyopyknosis, chromatin margination, aggregation, and scattered apoptotic bodies were observed. The results showed that 48 hrs after transfection, the apoptotic rate of the co-transfected group was 52.257%±4.677% and was significantly higher than those in the Livin group (42.778%±4.312%) and Survivin group (36.687%±3.912%) ($p<0.05$).

Discussion

RNAi is used in gene silencing, in which a specific double-stranded RNA, composed of sense and antisense RNA, is transferred into a cell to degrade the corresponding mRNA and thus to silence the target gene via a post-transcriptional gene silencing mechanism. RNAi can specifically and efficiently block gene expression. Thus the use of RNAi is an economic, efficient, and effective technique for gene function research. Moreover, the RNAi technique has gradually evolved as a tool for gene therapy research on viral diseases, hereditary diseases, and cancer [15-17].

Livin and Survivin, which are members of the IAP family, both participate in the inhibition of cell apoptosis [18]. Livin is highly and specifically expressed in certain tumor tissues and tumor cell lines [19]. Silencing of Livin by a shRNA vector increases tumor cell apoptosis in glioma, lung cancer, and other tumor cells [19,20]. Meanwhile, Survivin expression is related to decreased tumor apoptotic index, increased transfer rate, shortened overall survival, poor prognosis, and increased relapse rate [21]. The application of shRNA expression vector transfection to ovarian and lung tumor cells can effectively silence the Survivin expression in tumor cells and thus induce tumor apoptosis [22,23].

In this study, we selected target gene sequences from Livin and Survivin mRNA to construct corresponding shRNA eukaryotic expression vectors. The relative mRNA and protein expression were then analyzed using real-time quantitative PCR and Western blotting. The results showed that the mRNA and protein expression in the A549 cells from the co-transfected group were significantly reduced compared with that in the single transfection group ($p<0.05$). MTF results indicated that the growth inhibition rate was significantly high-

Table 1. Growth inhibition rate (%) of A549 lung cancer cells at different times after transfection of each group detected by MTT assay

Group	*48hrs*	*60hrs*	*72hrs*
Control	10.893±0.173	7.282±0.456	6.093±0.343
Livin	18.567±0.480[a]	14.279±0.762[a]	9.703±1.023[a]
Survivin	17. 971±0.642[a]	13.833±0.663[a]	9.430±0.821[a]
Co-transfection	28.043±0.762[ab]	22.193±0.127[ab]	15.093±1.140[ab]

[a]p<0.05 vs the control group; [b]p<0.05 vs the Livin and Survivin group

er in the co-transfection group than in the single-transfection group in the A549 cells. TUNEL assay confirmed that the apoptotic rate of A549 cells increased significantly in the co-transfected group compared with that in the single-transfected group. These results indicate that, compared with single-gene suppression, co-transfection of pSilencer-Livin and pSilencer-Survivin can effectively reduce the Livin and Survivin gene expression in A549 cells and promote A549 apoptosis. In addition, real time quantitative PCR results showed that the inhibition of a gene from the IAP family alone does not promote the expression of another gene. This finding indicates that Livin and Survivin expression may be independent of one another. However, the significantly reduced cell growth inhibition rates and increased apoptosis rate in the co-transfected group compared with those in the single-transfected group indirectly illustrate the synergistic effects of Livin and Survivin on the inhibition of apoptotic mechanisms.

This study confirmed that co-transfection can more effectively inhibit tumor gene expression in A549 cells and induce apoptosis. However, further studies must be conducted on the mechanisms of interaction and the specific binding sites of Livin and Survivin on a molecular level, such as in the apoptotic pathway.

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