Survivin expression in human lung cancer and the influence of its downregulation on the biological behavior of human lung cancer cells

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Abstract. The aim of the present study was to detect the expression of survivin in human lung cancer and to investigate the influence of its downregulation on the biological behavior of A549 lung cancer cells. The high expression of survivin in human lung cancer was verified by immunohistochemistry. Survivin small interfering RNA (siRNA) and unrelated sequence were synthesized and the siRNA lentiviral vector was constructed. The vector was transfected into A549 lung cancer cells, in which the clone with stable expression was screened out. We blocked the expression of survivin mRNA and protein by RNA interference (RNAi) technique. The downregulation of survivin mRNA and protein expression was confirmed by real-time quantitative PCR and western blotting. The proliferative activity and growth rate of A549 cells were determined by colony formation assay and mononuclear cell direct cytotoxicity assay (MTT assay). The reconstituted basement membrane (RBM) penetrating capacity was determined by cell invasion assay. The cell movement and migratory capacity were detected by wound-healing repair assay. The results showed that the sequence-specific siRNA significantly downregulated the expression of survivin at both the mRNA and protein levels. Downregulation of survivin expression dramatically decreased the invasive and metastatic capacities of the cells, suppressed the proliferation, decelerated the rate of growth, reduced the number of clones on soft agar and decreased the capacity of RBM penetration and migration. In conclusion, survivin, which plays an important role in carcinogenesis and development of lung cancer, can be effectively downregulated using the RNAi technique.

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Introduction

Survivin, a new member of the inhibitor of apoptosis protein (IAP) family, both inhibits apoptosis and regulates the cell cycle. It is overexpressed in most tumor tissues, but hardly expressed in most normal tissues. Therefore, it may be an attractive target for antitumor gene therapy (1-3). As an important technique for the research on gene function, RNA interference (RNAi) technique is an economical, fast and highly efficient technique for knocking down gene expression (4,5). In the present study, we constructed a survivin small interfering RNA (siRNA) lentiviral vector, assessed its effect on malignant biological behaviors (proliferation, invasion and metastasis) of lung cancer and verified the function of survivin in the carcinogenesis and development of lung cancer, in order to carry out further research on the mechanisms of this gene.

Materials and methods

Materials. A549 and 293T lung cancer cell strains were purchased from the Shanghai Cell Resource Center of the Chinese Academy of Sciences. pGC-LV vector, pHelper 1.0 vector and pHelper 2.0 vector were purchased from Shanghai GeneChem Co., Ltd. Qiagen plasmid Midi kit was purchased from USA Qiagen Co. tryptase was purchased from Shanghai Chemical Reagent Co., Ltd. *E. coli* DH5α, SYBR Master Mixture, T4 DNA ligase and *Taq* DNA polymerase were purchased from Takara Co. (Japan). *Age*I and *Eco*RI restriction enzymes were purchased from New England Biolabs (NEB) Co. Liposome Lipofectamine 2000 was purchased from Invitrogen Co (USA). DMSO was purchased from Shanghai Biological Reagent Co., Ltd. DMEM culture medium was purchased from Gibco Co. (USA). FBS was purchased from Shanghai Weike Biochemical Reagent Co., Ltd. Oligo(dT) was purchased from Sangon Biotech (Shanghai) Co., Ltd. M-MLV reverse transcriptase and dNTP were purchased from Promega Co. (USA). Rabbit survivin monoclonal antibody against the

human was purchased from CTS (USA). The S-P IHC kit was purchased from Fuzhou Maixin Bio Co., Ltd.

Thirty lung cancer specimen and corresponding surgical margin specimens were obtained from the Fujian Medical University Union Hospital.

Detection of the expression of survivin in tissue specimens by immunohistochemistry (IHC). The paraffin specimens were routinely dewaxed. Antibodies were fixated by microwave. Endogenous hydrogen peroxidase was blocked by incubation in 3% H_2O_2 for 10 min. The specimens were then incubated with rabbit survivin monoclonal antibody against human for 2 h at 37°C. After rinsing with PBS, the specimens were incubated with goat antibody against rabbit labeled with biotin for 15 min at room temperature. The specimens were rinsed with PBS again, and were further incubated with streptavidin-hydrogen peroxidase for 15 min at room temperature. After rinsing with PBS, the specimens were processed with DAB. Then, the specimens were redyed with hematoxylin, transparented, sealed and observed under a light microscope. Tissue positive for survivin was used as the positive control, and specimens for which PBS replaced the primary antibody were used as the negative control.

Construction of survivin-siRNA lentiviral vector and screening. We designed the target sequence according to the survivin mRNA sequence in GenBank and the principles of siRNA design. Four pairs of siRNA targeted with survivin and one pair of siRNA with negative control were designed (Table I). The synthesis of siRNA was carried out by the Shanghai GeneChem Co., Ltd. (6,7). The siRNA was subsequently transfected into 293T cells according to the guidelines for Lipofectamine 2000 from Invitrogen. The transfection results were observed under a fluorescence microscope 24 h later. The cells were collected 36 h later. At the same time, the protein was extracted. The most efficient siRNA was chosen by western blotting. The result showed that the first pair of siRNA was the most efficient. Double-stranded DNA fragment, with cohesive termini of the *Age*I and *Eco*RI restriction enzymes, and the hairpin sequence of 5'-GGCTGGCTT CATCCACTGCTTCAAGAGAGCAGTGGATGAAGCCAG CC-3' inside, was synthesized *in vitro*. The fragment was ligated into pGC-LV, and then transfected into *E. coli* DH5α. After amplifying and screening, the construction was confirmed successful by sequencing. The plasmid was extracted, and the survivin-siRNA lentiviral vector was recombined. The A549 lung cancer cells transfected with the survivin-siRNA lentiviral vector were considered the knockdown group (KD). The cells with the negative control sequence were considered to be the negative control (NC) and the cells with no sequence were considered as the control group (CON).

Detection of the expression of survivin mRNA by real-time quantitative PCR (RT-qPCR) test. Total RNA was extracted by TRIzol and reverse-transcripted into cDNA. Then, the RNA was detected by RT-qPCR. Survivin primer and actin primer (as internal control) were synthesized by the Shanghai GeneChem Co., Ltd. The sequences are shown in Table II. The reaction conditions of PCR were as follows: pre-denaturation

Table I. siRNA sequence-specific to survivin.

siRNA no.	Sequence
Survivin 1	GGCTGGCTTCATCCACTGC
Survivin 2	GGACCACCGCATCTCTACA
Survivin 3	GAAAGTGCGCCGTGCCATC
Negative control	TTCTCCGAACGTGTCACGT

Table II. Primer sequences of survivin and actin.

Gene	Primer (5'-3')	Product size (bp)
Survivin		113
Forward	ACCGCATCTCTACATTCAAG	
Reverse	CAAGTCTGGCTCGTTCTC	
Actin		302
Forward	GTGGACATCCGCAAAGAC	
Reverse	AAAGGGTGTAACGCAACTA	

at 95°C for 15 sec; denaturation at 95°C for 5 sec; annealing at 60°C for 30 sec; 45 cycles were completed. The mixture was denatured for 1 min at the end of the PCR, and then cooled to 55°C, at which the double strands of DNA are able to combine sufficiently. From 55 to 95°C the light absorption value was recorded for 4 sec at every 0.5°C. From this step, the melting curve was depicted. Quantitative analysis was performed using the ratio of the target gene to actin.

Detection of the protein expression of survivin by western blotting. Total protein of the A549 lung cancer cells was isolated 72 h after transfection. Protein quantification was performed by the BCA assay. The protein sample was normalized at the same time. The sample load was 30 µg total protein per lane. Protein from 10% SDS-PAGE gel was transferred to a PVDF membrane after electrophoresis. The protein was blocked with 5% nonfat dry milk at 4°C. Then, the primary antibodies, rabbit monoclonal anti-survivin (1:1,000) and anti-GAPDH (1:1,000) antibodies were added, respectively, and the mixture was incubated overnight at 4°C on a rocking platform. After washing, the membrane was added together with the HRP-conjugated secondary antibody (1:5,000) and incubated for 2 h. The membrane was then developed with ECL enhanced chemiluminescence system and exposed to X-ray film. Its gray scales were scanned by an image analytical system.

Detection of lung cancer cell proliferation by colony formation assay. Cells at a log phase of growth for each group were digested with 0.25% tryptase into a single-cell suspension, diluted and inoculated into 24-well plates at 200 cells/well, 3 wells/group. The cells were then incubated for 2 weeks. The incubation was terminated when visible clones formed on the plates. The clones were then washed with PBS. Paraformaldehyde (1 ml) was added and cells were fixated

for 30-60 min. The clones were washed with PBS again and then dyed with Giemsa for 20 min. The number of colonies in which there were >50 cells was counted under a microscope. The colony formation rate = number of colonies/number of incubating cells x 100%.

Determination of the lung cancer cell proliferation curve by MTT assay. The cells at a log phase of growth for each group were inoculated into 96-well plates at 100 μl/well. Ten microliters of MTT (5 mg/ml) was added to each well before termination. The plates were incubated at 37˚C in 5% CO_2 for 4 h. The supernatant was discarded. DMSO (100 μl/well) was added. The mixtures were shaken gently in order to dissolve the hyacinth in sediment. The absorbance (A) value at a wavelength of 570 nm was detected by a microplate spectrophotometer, and the suppression rate of cell proliferation was then calculated. Five wells were performed for each group. The suppression rate of proliferation of the lung cancer A549 cells = (1 - A value of KD)/A value of CON x 100%. Detection was carried out continuously for 5 days. The cell proliferation curve was sketched in order to compare the cell proliferation rate for each group.

Detection of the invasive capacity of lung cancer cells by cell invasion assay. The invasion chamber was put into an incubator. Warm serum-free medium (300 μl) was added into the insert. ECM was rehydrated for 1-2 h at room temperature. Medium was carefully removed from the insert. Medium (500 μl) rich in FBS was added to the lower chamber. A cell suspension (300 μl) was added into each insert. The insert was incubated for 72 h. Medium and non-invasive cells were removed. Dye (500 μl) was added into the empty wells of the plates. The insert was immersed in the dye for 20 min. The lower membrane surface of invasive cells was dyed. The insert was then immersed in a large cup, washed several times and dried in air. Images were captured under a microscope. The membrane was dissolved with 10% acetate and detected at OD570.

Detection of the cell migratory capacity by wound-healing repair assay. We scratched horizontal lines cross the wells at the back of 96-well plate with a marker pen. There were at least 2 lines for each well. Approximately 5x10^4 cells were added into each well. The next day, lines perpendicular to the horizontal lines were scratched with the head of a pipette. Cells were washed twice with PBS, and the dead skin cells were removed. Serum-free medium was added into the wells. The cells were incubated for 24 h. Images were captured under a fluorescent microscope. We replaced the medium with complete medium and further incubation in an incubator was carried out for 20 and 26 h. Images were captured to observe the cell distribution at the scratch zone at different times. The vertical distance in the inner face of the scratch zone was measured. The wound-healing repair rate = (vertical distance of the inner face of the scratch zone before repair - vertical distance of the inner face of the scratch zone after repair)/ vertical distance of the inner face of the scratch zone before repair x 100%.

Statistical analysis. Data were processed using SPSS15.0 statistical software. Quantitative data were expressed as the means ± standard deviation. One-way ANOVA was performed for comparison between different groups. Dunnett's t (when homogeneity of variances existed) or Dunnett T3 (when heterogeneity of variances existed) was calculated. The difference was significant at P-values <0.05.

Results

Expression of survivin in lung cancer. IHC results showed that the total positive rate of survivin expression in non-small cell lung carcinoma (NSCLC) was significantly higher than that in the paraneoplastic tissue (66.7 vs. 3.3%, P<0.05) (Fig. 1A and B).

Effect of siRNA on the expression of survivin in lung cancer cells. Western blotting confirmed that the first pair of siRNA was the most efficient, and this was ascertained to be the most competitive candidate to be recombined into the survivin-siRNA lentiviral vector (Fig. 2A). As shown by RT-qPCR, the expression of survivin mRNA in KD was significantly lower compared to that in NC and CON (P<0.05), and the difference between NC and CON was not significant. The result proved that survivin-siRNA was successfully transfected into A549 lung cancer cells, and it specifically downregulated the expression of survivin mRNA (Fig. 2B).

Effect of survivin-siRNA on lung cancer cell proliferation. Two hundred cells were inoculated and then incubated for 2 weeks. The result showed that the colony formation rates in CON, NC and KD were 60±6, 48±3 and 3±1%, respectively. The colony formation rate in KD was significantly lower than the rates in CON and NC (P<0.05), which indicated that the cells in KD had extremely low proliferating capacity (Fig. 3A). The cell proliferation curves were sketched based on the OD values in each group from Day 1 to 5. The initial OD values were CON: 0.18±0.02, NC: 0.17±0.01, KD: 0.16±0.01. The differences were not significant (P>0.05). However, the OD values on Day 5 proved that the difference between CON and NC was not significant (1.44±0.01 vs. 1.13±0.44, P>0.05), but the proliferative activity of KD (0.80±0.03) was significantly decreased (P<0.05). The OD values in the KD group on Days 3, 4 and 5 were 36.0, 43.1 and 44.6%. The result proved that the cell proliferation was markedly suppressed after transfection with survivin-siRNA (Fig. 3B).

Change in the invasive capacity and cell migratory capacity of the lung cancer cells after survivin interference. Reconstituted basement membrane penetrating capacity of A549 lung cancer cells reflected its invasive capacity in the Transwell test. The OD value 72 h later was 1.07±0.01 in KD, which was significantly lower compared to CON (2.53±0.2) and NC (1.96±0.1, P<0.05). The result proved that the invasive capacity of A549 cells significantly decreased after transfection with survivin-siRNA (Fig. 4A). The wound-healing repair rate at 20 h in the KD group was significantly lower than that in CON (61.3±3.4%) and NC (59.3±4.1%, P<0.05). The differences were more significant 26 h later, after scratching, whereas the difference between NC and CON was not significant (P>0.05). The result proved that the cell migration capacity decreased markedly after transfection with survivin-siRNA (Fig. 4B).
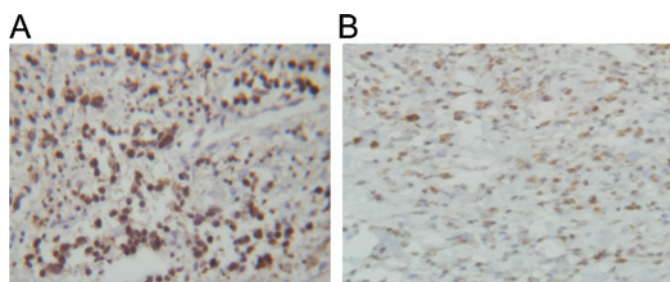
Figure 1. Expression of survivin in non-small cell lung carcinoma (A) Expression of survivin in lung cancer. (B) Expression of survivin in paraneoplastic tissue. Magnification, x40..
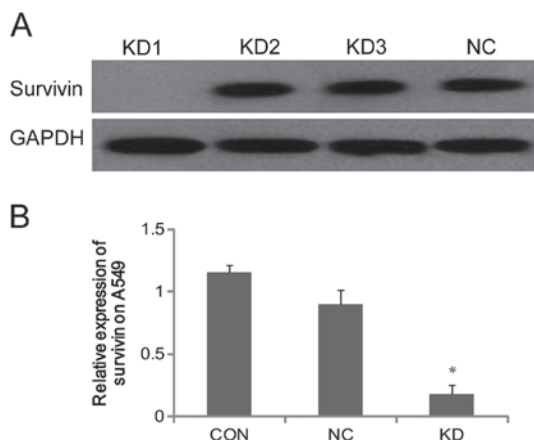

Figure 2. Downregulation of the expression of survivin in A549 cells. (A) Survivin protein was downregulation by four pairs of siRNA. (B) Effect of survivin-siRNA on the expression of survivin mRNA.
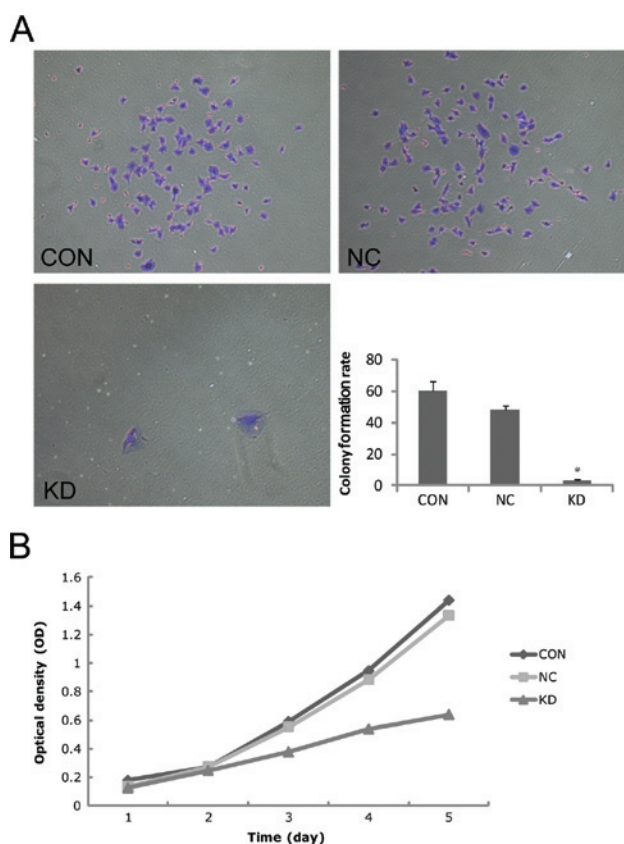

Figure 3. Effect of survivin-siRNA on lung cancer cell proliferation. (A) The cell clone in KD was severely inhibited as noted by Giemsa staining. (B) The MTT assay showed that cell proliferation decreased in the KD group.
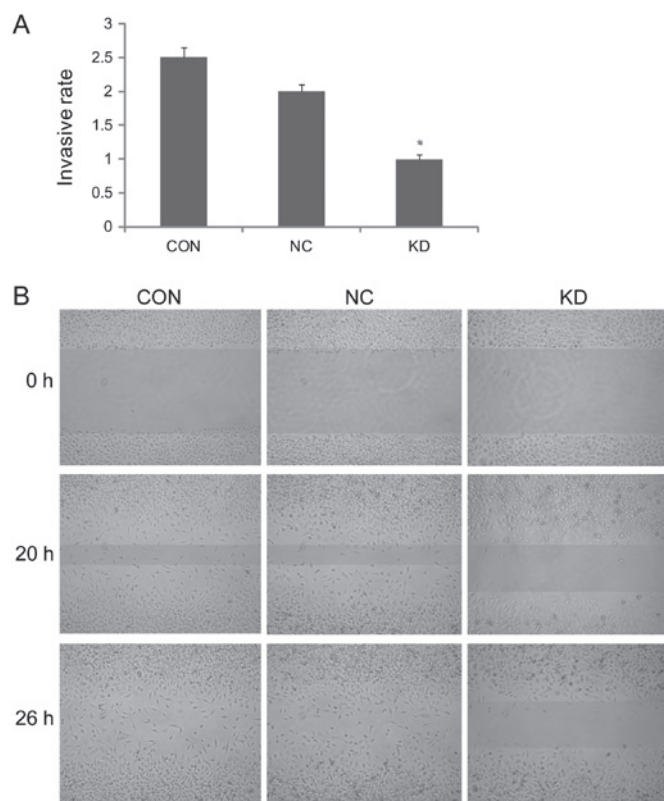

Figure 4. Change in the invasive and cell migratory capacities of lung cancer cells after survivin interference. (A) The invasive capacity significantly decreased in the KD group 72 h after survivin interference. (B) Wound-healing repair assay of each group 20 and 26 h after scratching.

Discussion

Lung cancer, one of the most malignant, health-threatening tumors, responds poorly to existing treatments and has an unfavorable prognosis. The identification of targets for the gene therapy of lung cancer has been an intense focus of research (8,9). Previous studies have shown that survivin is highly expressed in many types of tumors, and it directly or indirectly participates in carcinogenesis and development of tumors (10,11).

In the present study, we detected a high expression of survivin in tissue specimens of lung cancer by IHC, which was consistent with the research of Hofmann *et al* and Akyürek *et al* (12,13). The high expression of survivin in lung cancer offers a new method for its diagnosis. In our study, we used RNAi technique to suppress the expression of survivin to explore its importance, role and functions. Under this condition, we observed how survivin participates in the proliferation, growth, invasion and migration of lung cancer cells. The RNAi technique, the most effective antisense technique to date originated from a hereditary phenomenon widely existing in flora and fauna, and refers to a protective mechanism against gene instability caused by viral infection and insertion mutations. The technique specifically induces the degradation of target mRNA by double-strand siRNA. Compared to other gene knockout techniques, this technique shows high efficiency, specificity, stability, transmissibility

and hereditability, therefore it plays an important role in gene function research and gene therapy of tumors.

In our study, RT-qPCR and western blotting confimed that survivin siRNA effectively suppressed the expression of survivin in A549 lung cancer cells. In addition, the colony formation assay and mononuclear cell direct cytotoxicity assay proved that the survivin-siRNA sequence altered the proliferation and growth of the cells. In particular, the suppression rate of lung cancer A549 cell proliferation was 32.2%. In brief, our study proved the importance of survivin in maintaining and promoting the proliferation and growth of lung cancer cells at the mRNA and protein levels. After the transfection of survivin siRNA into lung cancer cells, the invasion and migration capacities were significantly altered, as shown by the markedly decreased cell membrane-penetrating capacity and wound-healing repair rate. All of the data proved the importance of survivin in invasion and migration of lung cancer. Dohi et al (14) proved that survivin could enhance the invasive capacity of tumor cells, independent of its anti-apoptosis capacity. Mehrotra et al (15) knocked out the expression of survivin and XIAP, two members of the IAPs, from invasive breast cancer MDA-MB-231 cells and prostate cancer PC3 cells using RNAi technique. They found that the invasive capacities of the two types of cancer cells markedly decreased. The same phenomenon was observed in intestinal cancer HCT116 cells. However, the invasive capacity of non-invasive MCF-7 cells was enhanced markedly after the cells were transfected with survivin. The animal models (mice) showed that the intermolecular cooperation between survivin and XIAP activated NF-κB, independent of IAP inhibition of cell death, which in turn led to increased fibronectin gene expression, signaling by β1 integrins, and activation of cell motility kinases FAK and Src. In this way, the invasion and migration of tumor cells were promoted. The researchers considered that antagonists against IAPs may provide anti-metastatic activity in patients with cancer.

In brief, downregulation of the expression of survivin may effectively suppress the malignant biological behavior of lung cancer, but more details regarding the mechanisms require further research (16-19).

Acknowledgements

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