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Original Article

Local injection of lentivirus-delivered livinshRNA suppresses lung adenocarcinoma growth by inducing a G0/G1 phase cell cycle arrest

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Abstract: The inhibitor of apoptosis protein (IAP) plays an important role in tumorigenesis and may be a potential target for cancer therapy. Livin, which belongs to this family, is highly expressed in various tumors. The previous study demonstrated that silencing Livin gene promoted lung cancer cell apoptosis; however, the effects on tumor growth suppression by targeting this gene in vivo, to thereby determine the efficacy of targeting Livin for patient therapy, have not been determined. This study injected lentivirus-delivered livinshRNA into established xenograft tumors derived from the lung adenocarcinoma cell line SPC-A-1 in BALB/C nude mice, the result showed that LivinshRNA down-regulated Livin expression effectively, induced tumor cell apoptosis, reduced tumor cell proliferation, and suppressed tumor growth dramatically, with a tumor volume inhibitory rate of (58.65±4.82)% and a tumor weight inhibitory rate of (47.44±1.64)%, but with less severe adverse reaction to the mouse. This study further demonstrated that Livin gene silencing induced a G0/G1-phase cell cycle arrest and cyclin D1 downregulation, which is a key regulator of the G0/G1- to S-phase transition. These findings suggest that LivinshRNA local injection may serve as a therapeutic method for patient treatment, and that LivinshRNA may suppress tumor growth by arresting the cell cycle in the G0/G1-phase.

Keywords: Livin gene, RNA interference, lung adenocarcinoma, xenograft tumor model, cell cycle

Introduction

Inhibitor of apoptosis proteins(IAPs), which can promote tumorigenesis by inhibiting apoptosis, are frequently suggested as targets for cancer therapy, and some IAP inhibitors have progressed to a preclinical stage [1-4]. Livin, a novel member of IAP family, is undetectable in most normal differentiated tissues but shows a high level of expression in a wide variety of human malignancies. Elevated activity of this protein is regarded as a predictor of poor prognosis [5–11] in cancer patients and a prognostic factor for resistance to chemotherapeutic agents or radioactive drugs in several cancers [6, 8, 11-15]. The inhibition of Livin gene expression in vitro was found to promote tumor cell apoptosis and sensitize tumor cells to different treatments [13, 14]. Although, collectively, these findings suggest that Livin may be a new effective target for cancer therapy [16–18], few in vivo anticancer experiments have been reported. Therefor, the present study further investigates the treatment effect of local injection of livinshRNA into lung adenocarcinoma.

Recent studies demonstrated that down-regulation of Livin expression by small interfering RNAs (siRNA) is effective in sensitizing cancer cells to treatment modalities and may emerge as a promising new therapy. Lentiviral delivery of interfering RNA (RNAi) can effectively and steadily silence gene expression, which can remedy the problem of the short half-life of these molecules in other RNAi systems [19]. Thus, lentiviral delivery of RNAi candidate drugs should overcome problems related to chemical stability and drug delivery and may be used as therapeutic agents. In this study, we introduced Livin shRNA via lentiviruses into the tumors of

lung adenocarcinoma xenograft models to down-regulate Livin expression and investigate the effect of this treatment in vivo.

The exact mechanism of the growth-promoting effects of IAP on tumors remains unclear. Studies have suggested that IAP abrogates apoptosis by binding to caspase or by down-regulating the JNK1 pathway [10, 20]. Recent studies found that IAP up-regulates cyclin expression and argue that this family may affect the regulation of the cell cycle to affect tumor growth [21-23]. As our previous study demonstrated that Livin gene silencing decreased tumor cell proliferation, we hypothesized that Livin may play a role in the regulation of the cell cycle [24]. This study attempts to further probe the effects of this gene on the cell cycle during tumor growth.

Materials and methods

All of the procedures were performed in accordance with the Declaration of Helsinki and relevant policies in China.

Cell lines and culture condition

The human lung adenocarcinoma SPC-A-1 cell line (Nan Jing Key Gen Biotech Co. Ltd, China) was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (Gibco company, USA), 100 unit/ml penicillin and 100 mg/L streptomycin. This cell line was incubated at 37°C in a humidified 5%CO^2/95% ambient air atmosphere.

Construction of a livin shRNA expression vector and its transfection

We used the dsRNA sequences corresponding to the 786-804bp locus of the Livin β cDNA, which had been confirmed to be an effective target due to homology to 2 Livin mRNA isomers in our previous study [20]. The sense strand was 5'-CAGGAGAGAGGTCCAGTCTGA-3', and the antisense strand was 5'-TCAGACTGGACCTCTCTCCTG-3'. The negative control shRNA targeted a nonspecific sequence. The shRNAs were subcloned into a lentiviral vector as described in a previous study [20] (Lentivirus- delivered shRNA was synthesized and constructed by Neuron Biotech company, Shanghai, China).

Establishment of a lung adenocarcinoma xenograft model in nude mice

A total of 18 BALB/C Nude Mice, including 9 females and 9 males, weighing 16-18 g and aged 4 to 6 weeks were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Science [Certificate of Quality No.: SCXK (Shanghai) 2007-0005] and maintained in specific pathogen-free facilities at the Experimental Animal Center of Fujian Medical University. The experimental procedures were performed according to the guidelines of this center. SPC-A-1 cells in logarithmic growth phase were collected and digested by 0.25% pancreatic enzyme-ethylene diaminetetraacetic acid (EDTA) digestive fluids. A single-cell suspension was suspended in serum-free culture medium. A total of 1×10^6 cells were injected into the skin of the right armpit of 18 nude mice. On the 8th day after inoculation, when transplantation tumors had grown to approximately 100 mm^3, the 18 nude mice were randomly divided into three groups: blank group (CON), negative group (NC) and experimental group (KD). Different interventions were performed on these three groups over four consecutive days: (1) blank group (CON): the tumors were injected with 100 μl normal sodium (NS); (2) negative group (NC): the tumors were injected with 2×10^6 TU (100 μl) lentivirus delivering non-specific sequence (LV-NC shRNA); (3) experimental group (KD): the tumors were injected with 2×10^6 TU lentivirus (100 μl) delivering Livin shRNA (LV-Livin shRNA).

Tumorigenicity studies

The tumor volume was measured as (long diameter × short $diameter^2/2$) every three days, and the growth curve was created with SPSS18.0 software. These three groups of nude mice were simultaneously sacrificed on the 26th day, and the tumors were removed and weighed. The inhibitory rates were calculated with the following formulas: the inhibitory rate of volume = (1- tumor volume of KD group/tumor volume of CON group) × 100%, and the inhibitory rate of weight = (1- tumor weight of KD group / tumor weight of CON group) × 100%.

Western blotting

One milliliter of cell lysis buffer was used per 1 mm^3. The buffer consisted of 100 mmol/l Tris

hydrochloric acid, 100 mmol/l sodium chloride, 0.5% sodium deoxycholate, 1 mmol/l ethylenediaminetetraacetic acid (EDTA), 1% NP40, 0.1% sodium dodecylsulfate (SDS) and protease inhibitor, with a pH of 7.5. The lysis procedure was as described in our previous study [20]. Primary antibodies consisted of rabbit polyclonal Livin antibody at a dilution of 1:1,000 (Imgenex company, USA), rabbit polyclonal Ki67 antibody at a dilution of 1:1,000 (Abcam company, USA), rabbit polyclonal cyclin D1 antibody at a dilution of 1:1000 (Abcam company, USA and mouse monoclonal β-actin antibody at a dilution of 1:1,000 (Santa-Cruz Company, USA). The secondary antibodies were at a dilution of 1:5,000 (Beijing zhong shan company, China). Equal amounts of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel for β-actin, Livin, and cyclin D1 and on 7.5% polyacrylamide gel for Ki67 protein. Electrophoresis was performed with a vertical electrophoresis apparatus (BioRad Company, USA) at 90V for 20 min and 120V for 2 h. The transfer of β-actin, Livin, cyclin D1 and Ki67 protein was performed with a semi-dry transfer apparatus (Phamacia Sweden TE-TO) for 2 h, 2 h, 2 h and 3 h, respectively. Transmembranes were incubated with primary antibodies at 4°C overnight and with secondary antibodies at 37°C for 1 h. Film development and exposure were performed with the ECL Plus kit (Amersham Company, China).

Immunochemistry assay

Five micrometer sections obtained from paraffin embedded tumor tissues were stained with Hematoxylin & Eosin for histological analysis or subjected to immunohistochemistry. Immunostaining was performed using the Polink-2 plus Polymer hematoxylin and photographed (HRP) Detection System. The sections were incubated with a primary rabbit polyclonal Livin antibody (Maixin Biocompany, China) at a dilution of 1:400 at 37°C for 1 h. Control immunostaining was performed with an IgG isotype alone. After rinsing three times, the sections were incubated with HRP Polymer at 37°C for 30 min. The final stain appeared after adding Diaminobenzidine (DAB) Plus Substrate.

TUNEL assay

TUNEL-positive tumor cells were detected using a TUNEL apoptosis detection kit according to the manufacturer's protocol. The paraffin-embedded slides were conventionally dewaxed and then incubated for 30 min with 0.25% parenzyme at 37°C for antigen repairing. The slides were rinsed and incubated at 37°C for 60 min with 50 μl of TUNEL reaction mixture and then rinsed again and incubated with 50 μl of peroxidase (POD) at 37°C for 30 min. Finally, the slides were stained with DAB and hematoxylin. The labeled solution was used as negative control. The cellular apoptotic index was calculated as follows: apoptosis index (AI) = (the number of positive cells / the number of total cells) × 100%.

Flow cytometry analysis

The fresh tumor tissues were prepared as single cell suspension. The level of apoptosis and cell cycle distribution were detected according to the manufacturer's instructions for the Annexin V-fluorescein isothiocyanate / Propidium Iodide (Annexin V-FITC/PI) kit (Nanjing Keygen Biotech. CO. LTD, China). The cells were harvested, washed with phosphate-buffered saline (PBS) twice and resuspended in binding buffer (10 mM Hepes pH7.4, 150 mM NaCl, 2.5 mM CaCl2, 1 mM MgCl2, 4% bovine serum albumin). AnnexinV-fluorescein isothiocyanate (0.5 mg/ml) and Propidium Iodide (PI, 0.6 mg/ml) were added to a 100-ml aliquot of the cell suspension with 2.0×10^6 cells. After a 20-min incubation in the dark at room temperature, the stained cells were immediately analyzed by flow cytometry (BD Company, USA). The cellular apoptotic rate was calculated with the following formula: the cellular apoptotic rate= (apoptotic cell number / total cell number) × 100%. Approximately 1.0×10^5 cells per group were seeded into a 6-well culture plate. Then cells were harvested by trypsinization after being cultured for 72 h, fixed with 70% ice-cold ethanol and stored for 48 h. The fixed cells were stained with PI. The analysis of cell-cycle distribution was performed using FAC Scan Cytometer (BD Company, USA) and analyzed using Cell Quest software.

MTT assay

The fresh tumor tissues were prepared as single cell suspension. Cells were seeded into six 96-well culture plates at 1×10^4 per well. Each group had 6 replicate wells. The absorption value (A value) was determined by MTT at 490

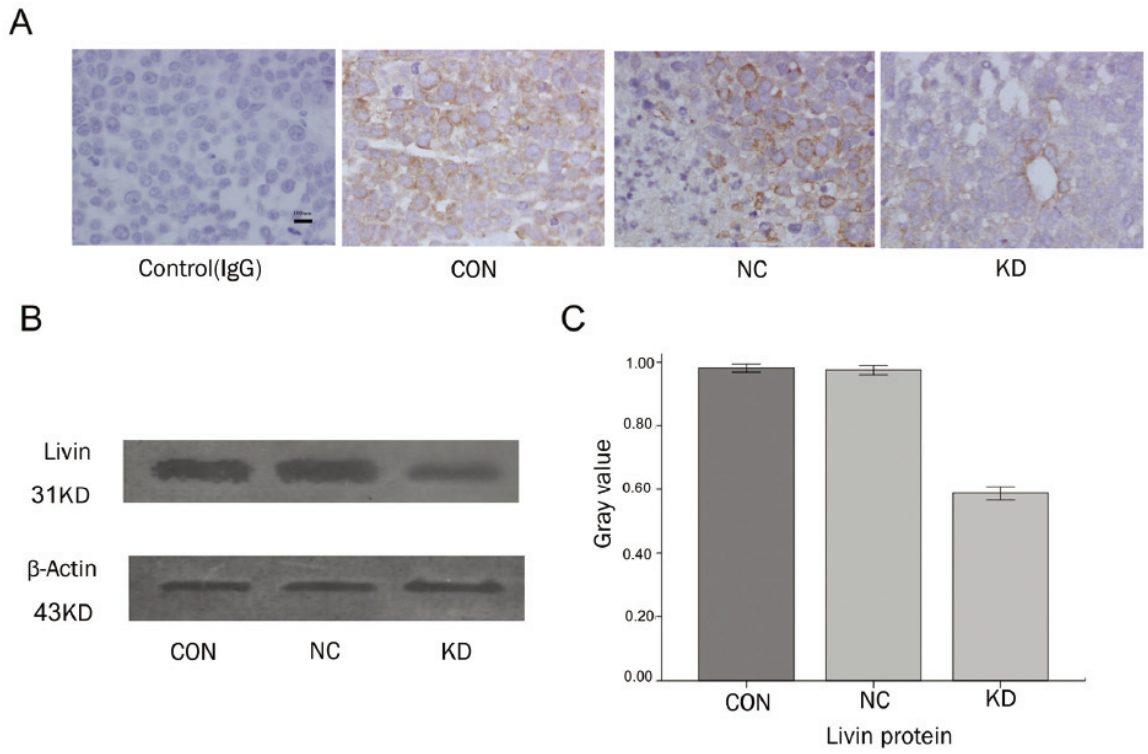

Figure 1. Local injection of lentivirus-delivered livinshRNA specifically silenced Livin gene expression in xenograft tumors. Livin expression was determined after lentivirus-delivered livinshRNA was injected into xenograft tumors derived from the lung adenocarcinoma cell line SPC-A-1 in BALB/C nude mice. A. Immunostaining of Livin protein in xenograft tumors of the three different groups: blank control (CON), negative control (NC) and experimental group with Livin gene silenced (KD). Slides were counterstained with hematoxylin and photographed using a light microscope. Original magnification is denoted by the scale bar in the control. B. Western blotting analysis of Livin expression in the three groups of xenograft tumors. C. Expression of Livin was quantified and normalized to the amount of β-actin gene expression, and the average of the three independent experiments was calculated.

nm after 24 h of cultivation. The absorption value of every culture plate was detected at the following times: 48 h, 72 h, and 96 h. The proliferation curve of each group was plotted on the basis of the absorption values. The inhibitory rates of cell proliferation were calculated according to the following formula: inhibitory rates of cell proliferation = (1- the A value of KD group / the A value of CON group) × 100%.

Statistical analysis

All experiments were performed in triplicate. One-way analysis of variance (ANOVA) was used for tumor weight, Livin and Ki67 expression, and the A value for MTT in the different groups. The repetitive measure analysis of variance was used for tumor volume in the three groups. Statistical analysis was performed using SPSS18.0 (2010 SPSS Inc.) software, with the significance cut-off set at $p < 0.05$.

Results

Local injection of lentivirus-delivered livinshRNA specifically silenced Livin gene expression in xenograft tumors

We subcloned the effective Livin shRNA into a lentiviral vector, and then injected lentiviruses expressing Livin shRNA into the tumors derived from the lung adenocarcinoma cell line SPC-A-1 in BALB/C nude mice at the proper optimum concentration according to our primary study (data not shown). As a result, Livin gene expression was specifically silenced in the xenograft tumors, with a silencing effect of greater than 50% (**Figure 1**).

Local injection of lentivirus-delivered livinshRNA suppressed tumorigenesis in nude mice

Previous studies have reported that the down-regulation of the Livin gene could induce cellu-

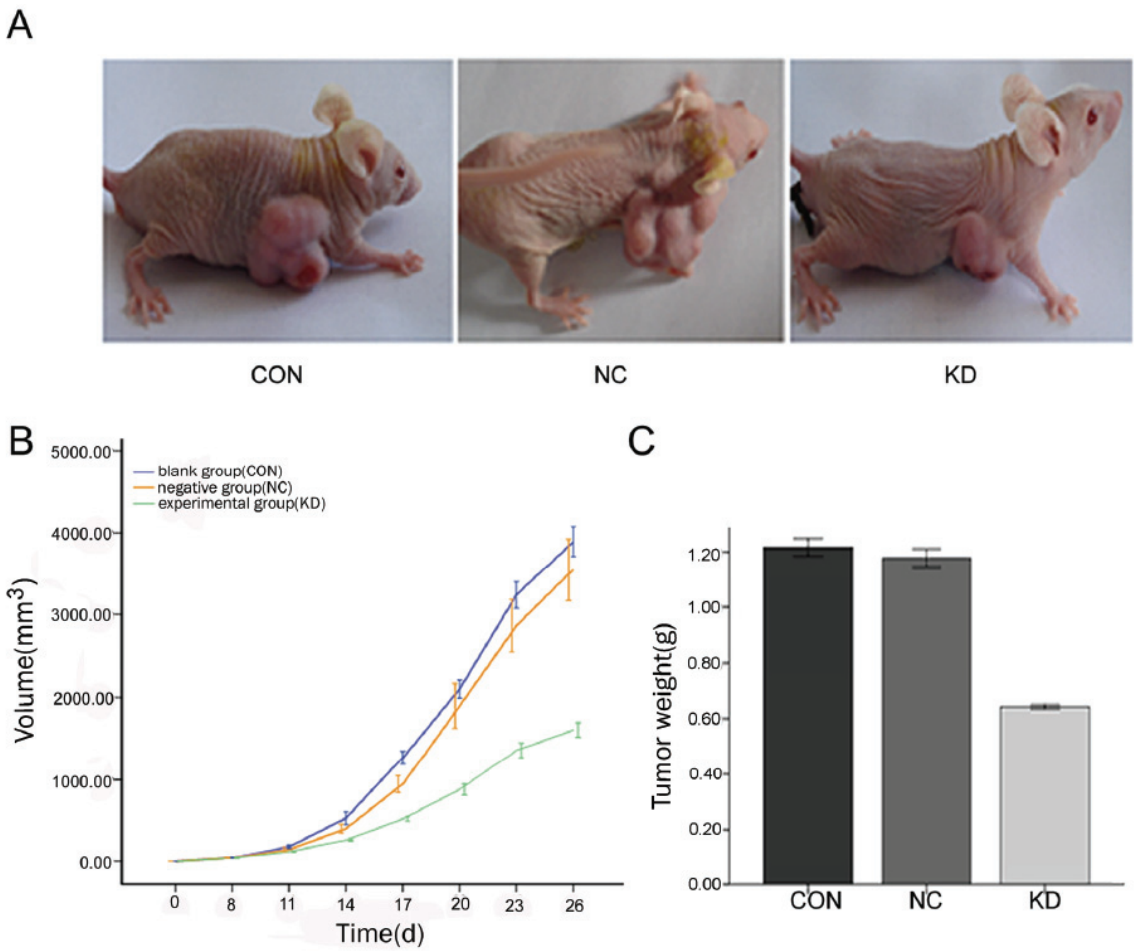

Figure 2. Local injection of lentivirus-delivered livinshRNA suppressed tumor growth in nude mice. A. On the 8th day after SPC-A1 inoculation, when xenograft tumors in the 18 BALB/C nude mice model had grown to approximately 100 mm^3, they were randomly divided into three groups: injected with NS (CON), lentivirus delivering non-specific shRNA (NC), and lentivirus delivering Livin shRNA (KD). Mice were examined and killed until tumor volume appeared to be different in three groups on the 26th day. B. Tumor volumes were closely observed every three days, recorded, and presented as a growth curve using the mean volumes ± SE. C. At the termination of the study, tumors were excised from each mouse and weighed. The wet weight of the tumors is represented as the mean of 6 tumors from individual mice in each group.

lar apoptosis in vitro. We further investigated whether silencing of this gene would also inhibit the tumorigenesis of pre-established SPC-A-1 transplantation tumors in vivo. We first examined the location of the primary tumors and their metastases and found no distal metastases in the three groups. Then we closely observed the tumor growth in these groups. Although the growth of the tumors was not completely suppressed by LV-Livin shRNA, the tumor volume and the growth rates of the KD group were significantly lower than those of the blank and negative control groups ($p<0.01$) (**Figure 2A-B**). Furthermore, the tumor weight of the KD group was significantly less than that of the control groups [(0.64±0.02)g versus (1.22±0.90)g and (1.16±0.04)g; $F=254.64$, $p<0.01$] (**Figure 2C**). There were no significant differences in either tumor volume or tumor weight between the CON group and the NC group ($p>0.05$). The overall reduction in tumor volume was (58.65±4.82)% (**Figure 2B**), and the reduction in tumor weight was (47.44±1.64)% (**Figure 2C**) following Livin gene silencing.

Local injection of lentivirus-delivered livinshRNA induced cellular apoptosis and decreased cell proliferation

We tested the effect of Local injection of LivinshRNA on tumor cellular apoptosis by

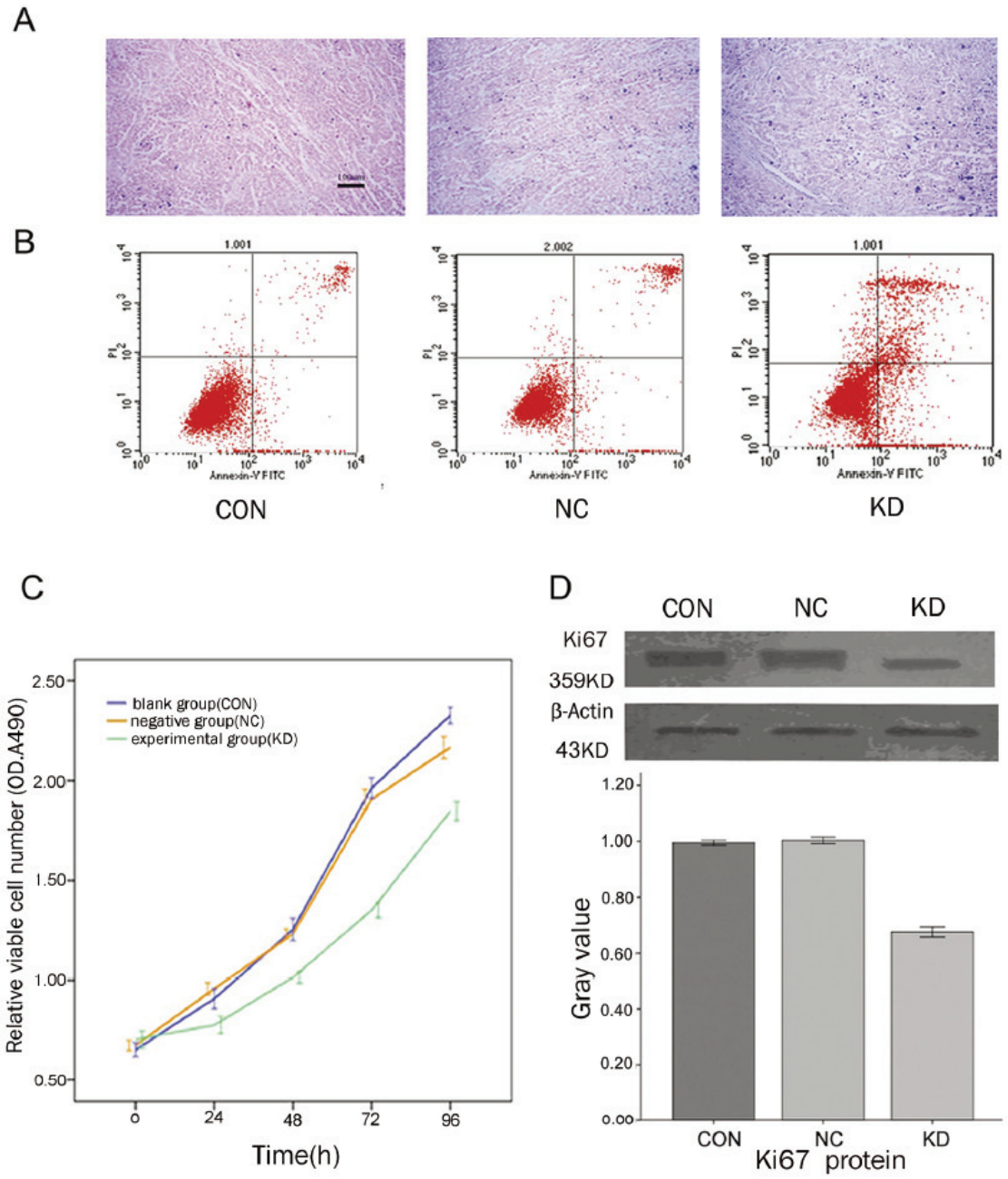

Figure 3. Local injection of lentivirus-delivered livinshRNA increased cellular apoptosis and decreased cellular proliferation. Xenograft tumors of three groups in BALB/C nude mice were removed on the 26th day, then their cellular apoptosis and proliferation were observed. A. The paraffin-embedded slides were conventionally dewaxed, and apoptosis was detected by the TUNEL assay. B. Cellular apoptosis was detected by staining with Annexin V-fluorescein isothiocyanate/Propidium Iodide (Annexin V-FITC/PI) and flow cytometry. Early apoptosis (LR, Annexin V+/PI−), late apoptosis (UR, Annexin V+/PI+), necrotic cells (UL, Annexin V−/PI+), and normal cells (LL, Annexin V−/PI−). C. Absorption values at 490 nm of the different groups were determined by MTT assays at 24 h, 48 h, 72 h, and 96 h post-seeding. The proliferation curve of each group was plotted on the basis of the absorption values. D. Ki67 protein expression was determined by western blot and quantified and normalized to the β-actin gene expression level, and the average of the three independent experiments was calculated.

TUNEL analysis and flow cytometry. TUNEL analysis revealed that tumor sections from mice treated with the LV-Livin shRNA had a higher percentage of apoptotic cells than did

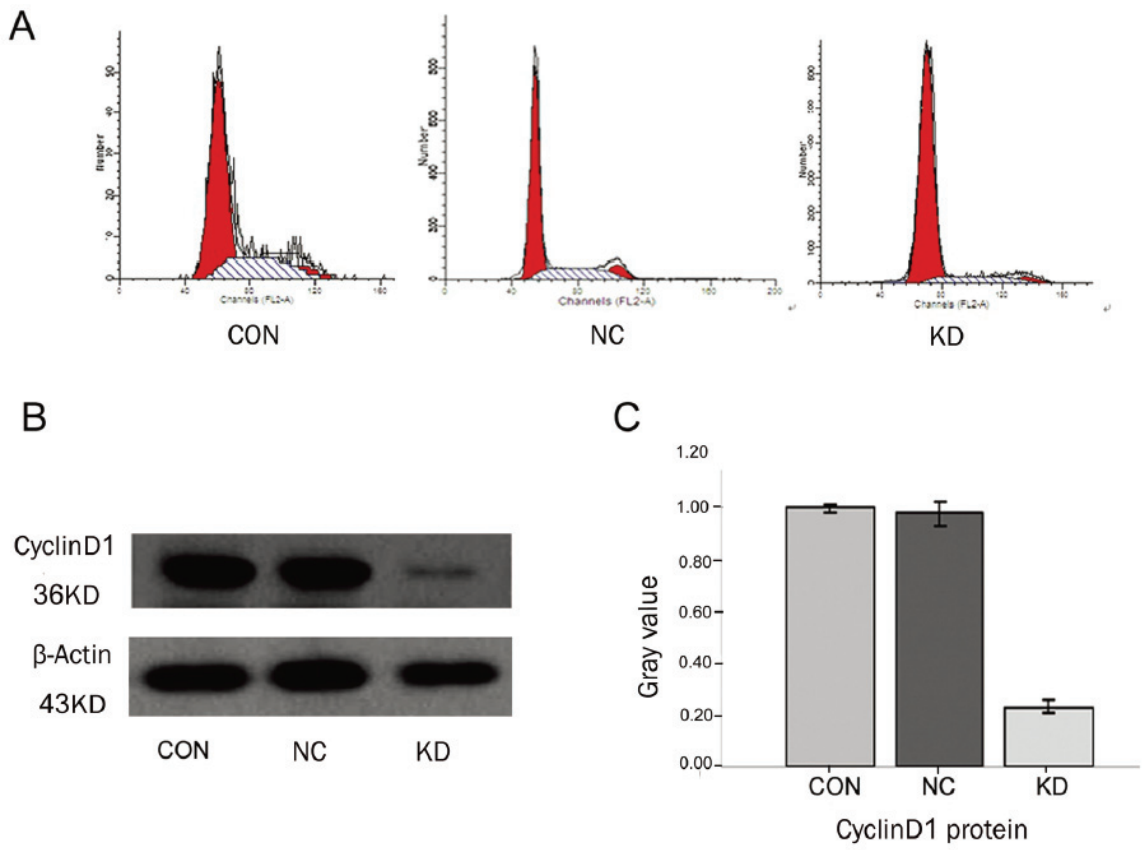

Figure 4. Livin gene downexpression induced cell cycle arrest and down-regulation of cyclin D1 expression. A. The cell cycle was detected in tumors of three groups by staining with Annexin V-fluorescein isothiocyanate/Propidium Iodide (Annexin V-FITC/PI) and analysis by flow cytometry. B. Then Cyclin D1 protein expression in tumors of these three groups was detected by a western blot assay. C. Cyc*lin D1* gene expression was quantified and normalized to the amount of *β-actin* gene expression, and the average of the three independent experiments was calculated.

those from controls [(31.48±3.79)% versus (6.55±1.30)% and (7.47±1.28)%; p<0.01] (**Figure 3A**). Flow cytometry also revealed a higher apoptotic rate in the KD group than that in CON and NC groups [(15.47±1.14)% versus (4.42±0.76)% and (5.26±1.02)%; p<0.01] (**Figure 3B**). Next, we tested the effect of Livin shRNA on cell proliferation using an MTT assay and by determination of ki67 expression. Analysis of the MTT assay showed a significantly higher inhibitory effect in the tumor tissues with the Livin gene silenced compared to the two controls at 24,48,72,and 96h of cultivation(p<0.01) (**Figure 3C**). The proliferation inhibitory rate was (31.15 ± 4.62)% and reached a peak at 72 h. Accordingly, western blot analysis revealed lower expression of Ki67 following Livin gene silencing (**Figure 3D** p<0.01). These results demonstrate that Livin gene silencing induces cellular apoptosis and decreases cell proliferation in vivo.

Livin gene down-expression induced cell cycle arrest and down-regulation of cyclin D1 expression

To reveal the effect of Livin gene silencing on the cell cycle during tumor growth, we first performed a cell-cycle analysis using flow cytometry in the three groups. We demonstrated that the population of cells in the G0/G1-phase of the cell cycle was significantly increased in the KD group when compared with those in the CON and NC groups [(89.67±6.33)% versus (67.05±6.26)% and (63.17±2.57)%, p<0.01]; however, the population of cells in the G2/S phase showed the opposite trend [(10.34±6.34)% versus (36.83±2.57)% and (32.96±6.25)%, P<0.01] (**Figure 4A**).

Furthermore, the expression of cyclin D1, a key regulator of G0/G1- to S-phase transition, was significantly lower in the KD group (**Figure 4B-C**). These results suggested that Livin gene silencing induced cell cycle arrest in G0/G1-phase.

Discussion

There have been eight human IAP molecules identified thus far: NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/ML-IAP (BIRC7), and IAP-like protein 2 (BIRC8) [25]. They are highly conserved from yeast to mammals,which suggests their importance for organismal viability [26]. The overexpression of the IAP family members in cancer tissues has been widely reported, and these proteins are suggested to be important targets for cancer treatment [3-4]. Livin was reported in 2001 as a novel IAP family protein [10], which differs from other IAPs in that it has only a BIR and a Ring domain. The Livin mRNA has two isoforms: α and β. Overexpression of this gene has been found in various tumors, including melanoma, breast, cervical, colon and prostate cancers, and leukemia [5-12]. Additionally, our previous study demonstrated that Livin was highly expressed in lung cancer tissues [27]. Livin silencing could induce apoptosis of lung adenocarcinoma cell line SPC-A-1 in vitro [20]. Base on the research, we further investigated the effect of local injection of lentivirus-delivered livinshRNA as a therapeutic method in this study.

In this study, we used lentiviral delivery of Livin shRNA (LV-Livin shRNA) for local injection into the subcutaneously heterotopic transplantation tumor as an easy treatment method [28]. We obtained a sequence-specific silencing of two isoforms of the Livin gene using this method of targeting the 787-796bp locus of the Livin β mRNA, which shares homology with the Livin α mRNA [20]. We found that this delivery system stably and effectively knocked down Livin expression as described in vitro [20]. We used 2×10^6 TU of lentivirus to deliver the siRNA and gained an inhibition rate of >58% in our model, whereas Bo-Young Oh used 10-50 μmol/l of liposome-delivered siRNA in a colon cancer model. He regarded 20 μmol/l as the best dose, with a maximal inhibition rate of 50%, although he found the inhibition rate was neither dose-dependent nor time-dependent. However, further study is necessary to determine the quantity of siRNA necessary for cancer treatment.

In this study, we found no distal metastases to other organs in this model except that one third of the mice exhibited metastasis to the ipsilateral armpit lymph nodes and infiltration of local tissues. Furthermore, we examined the side effects of local injection of LV-Livin shRNA and found neither fatalities nor severe adverse reaction, with the exception of local necrosis, which is similar to Bo-Young Oh's study on colon cancer [28]. Because of the less severe adverse reaction of LV-Livin shRNA, we performed the injection for four consecutive days, which is distinct from other intervention experiments in which injections were performed every 2-3 days [29] or weekly [28]. This result demonstrates the safety of LV-Livin shRNA local injection and suggests that this method may be a promising treatment for cancer [19].

We also found that Livin gene silencing induced cell cycle arrest and decreased the proliferation of lung cancer cells, which was in agreement with the findings of Wang's study [29]. The expression of cyclin D1, which is required for the transition from the G1/G0- to S-phase of the cell cycle by binding to Cdk4/6 kinase [30], simultaneously decreased with Livin gene down-regulation in the tumors. We hypothesize that Livin may regulate the cell cycle of SPC-A1 cells by increasing cyclin D1 expression. Livin expression could promote cellular switching from the G1/G0-phase to the S-phase, thus beginning a new round of DNA synthesis and cell proliferation. Other IAP family members, such as survivin and c-IAP, have also been shown to control cell cycle progression [23, 31-33], specifically by regulating cell cycle checkpoints [23]. Connell et al. [23] showed in Hela cells that nuclear survivin facilitates S-phase entry by subversion of the G1-S checkpoint control. Nuclear survivin sensitizes cells to S-phase induction by promoting pRb inactivation, which allows elevated levels of free E2F to act as the potent transcriptional activators needed for the G1-S transition. Recently, the expression of cIAP1 in the nuclear compartment has also been documented to regulate the cell cycle [34]. Therefore, we propose that

Livin may enhance tumor growth by promoting cell cycle progression, in addition to the established mechanisms of caspase binding or JNK1 pathway regulation [10, 20].

In conclusion, The local injection of lentivirus-delivered LivinshRNA can suppress lung carcinoma tumor development effectively but has minor adverse reaction, LivinshRNA gene may decrease tumor growth by arresting cell cycle from G0/G1- to S-phase progression by down-regulate cyclin D1 expression.

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