

Synergistic Effects of Targeting Survivin and CDK1 on Nasopharyngeal Carcinoma *in Vitro* and *in Vivo*

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Abstract

Background: To explore the impact of pU6-based tandem survivin and CDK1-specific short hairpin RNA on the biological behaviors of CNE-2 nasopharyngeal carcinoma cells *in vitro* and *in vivo*. **Patients and Methods:** The vectors of pU6-survivin_{shRNA}, pU6-CDK1_{shRNA} and pU6-survivin_{shRNA}-CDK1_{shRNA} were constructed and transfected into CNE-2 cells with Lipofectamine TM 2000, respectively. The mRNAs and proteins of CDK1 and survivin were determined by RT-PCR and Western blotting, accordingly. MTT assay was employed to evaluate the proliferation of CNE-2 cells, and flow cytometry was performed to determine the apoptosis of CNE-2 cells. The effects of interfering survivin and CDK1 on tumorigenesis were evaluated by tumor xenografts experiments. **Results:** Effective plasmids were successfully constructed knocking down survivin and/or CDK1. The proliferation inhibition of CNE-2 cells by pU6-survivin_{shRNA}-CDK1_{shRNA} (32.5%) was higher than that of by pU6-survivin_{shRNA} (25.6%) and pU6-CDK1_{shRNA} (15.6%), and apoptosis in CNE-2 cells simultaneously interfering survivin and CDK1 (15.2%) dramatically increased when compared to those of interfering survivin (5.4%) or CDK1 (4.7%) alone. Furthermore, simultaneously interfering survivin and CDK1 is more effective than interfering alone component in inhibiting tumor growth of fBalb/C nude mice xenografted with CNE-2 cells. **Conclusion:** The results altogether indicate that interfering survivin and CDK1 simultaneously can produce synergistic effects of anti-nasopharyngeal carcinoma, which could be a potential therapeutic method.

Keywords

Surviving, Cyclin-Dependent Kinase1, RNA Interference, CNE-2

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1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most popular cancers in southern China [1]. Though NPC is sensitive to radiotherapy, the outcome of the treatment is depressing. The five year survival rate is only 50% - 60% due to factors below: most NPC organization cells is undifferentiated with high malignancy; the NPC anatomical location impedes radical resection and the remaining NPC cells serve as seeds for future recurrence and metastasis in future; NPC cells are insensitive to chemotherapy; NPC cells develop radio resistance during radiotherapy. Therefore, it is essential to develop a new therapeutic technology to improve the unsatisfactory outcome of NPC treatment. With the development of technology of gene recombination and transgenesis, RNA interference technology has become a powerful tool of knocking out targeted gene expression, which offers an optimistic treatment for various cancers.

Multiple up-regulated genes are involved in tumorigenesis and metastasis, so the treatment of targeting a single gene is generally unsatisfactory. Therefore, researchers attempt to solve the problem through two ways: targeting the nodal protein or targeting multiple proteins simultaneously. Survivin, a member of the inhibitor of apoptosis (IAP) family, is closely associated with many physiological and pathological processes, including interaction with multiple regulatory factors, modifiers and cellular networks [2] [3] [4] [5]. Survivin is highly expressed in almost all cancers, including NPC. However, expression of survivin is undetectable or rather low in almost all normal mature cells, which makes survivin an ideal target for various cancers [3] [4] [5] [6]. CDK1, an important member of controlling mitosis, plays a key role in G1/S and G2/M phase transitions of eukaryotic cell cycle [7] [8]. Moreover, CDK1 is also the enzyme for catalyzing the phosphorylation of 34 survivin threonine residue site [9]. Overexpression of CDK1 in cancers induces cell proliferation and chromosomal instability, which also makes CDK1 a potential therapeutic target [10] [11] [12]. Short hairpin RNAs (shRNA) is efficient in silencing specific gene in mammalian cell [13]. Therefore, the study is aimed at observing impacts of knocking out survivin and CDK1 simultaneously on nasopharyngeal carcinoma by constructing pU6-survivin_{shRNA}-CDK1_{shRNA}, and evaluating the potential value of survivin and CDK1 in tumor therapy.

2. Materials and Methods

2.1. Materials

pU6-M4 plasmid was kindly bestowed by Dolph L. Hatfield (National Institutes of Health, Maryland, United States). Other materials involved in the study were purchased from companies or corporations as below: competent Trans-5αE. coli (TransGen Biotech, Beijing, China), CNE-2 cells (China Center for Type Culture Collection), MEM medium and Opti-MEM (Gibco Corporation, Grand Island, NY), T4 DNA ligase and restriction endonucleases of BamHI, HindIII, Xho I, Mfe I and EcoR I (Thermo Fisher Scientific, Waltham, USA), rapid gel recovery

kit and plasmid maxi kit and RNA extraction kit (QIAGEN, Hilden, Germany), lipofectamineTM2000 transfection Reagent Kit (Invitrogen Corporation, Grand Island, NY), BioTakesupermo III RT Kit and quantitative fluorescence PCR kit (Bio TeKe Corporation, Beijing, China). MTT, trypsin, rabbit anti-human Survivin, rabbit anti-human CDK1 and horseradish peroxidase-labeled goat anti-mouse antibodies were all purchased from Sigma Corporation, Shanghai, China, while rabbit anti-human β -actin antibody purchased from Massachusettes, USA.

2.2. CNE-2 Culture

CNE-2 cells were cultured at 37°C in MEM medium supplemented with 10% FBS (HyClone), 100 U/mL penicillin, 100 mg/mL streptomycin (Invitrogen) in an incubator with 5% CO₂. The cells were digested with 0.25% trypsin for passage when grown to 85% - 90% confluence.

2.3. Generation of shRNA pU6-M4 Plasmids

Genbank accession number (survivinNM_001012271) and (CDK1 NM_001170406) were used for this study. Scrambled RNA sequence without significant homology to mouse and human gene sequences, was used as negative control to validate the specific effects. Terminator code (TTTTT), restriction sites of BamHI (G^ATCC) and HindIII (A^AGCCT) were added to where to form BamHI-sense-loop-anti-sense-terminator-HindIII. A series of plasmids containing shRNA specific to Survivin and CDK1 gene, either alone or in combination, were cloned into pU6-M4 vector by ligating the BamHI/HindIII-digested shRNA fragment to the vector after digestion by the same restriction endonucleases as described previously. The diagram of constructed pU6-survivin_{shRNA}-CDK1_{shRNA} was as shown in **Figure 1**.

2.4. Plasmids Transfected CNE-2 Cells

Constructed plasmids were transfected into competent Trans-5*aE.coli* according to the instructions of LipofectamineTM 2000 Transfection Reagent Kit. Seven groups were categorized according to the transfection: transfection with pU6-survivin; transfection with pU6-CDK1_{shRNA}; transfection with pU6-survivin_{shRNA}-CDK1_{shRNA}; transfection with pU6-survivin_{shRNA}-NC; transfection with pU6-CDK1_{shRNA}-NC; Lipofectamine 2000; control without adding anything. About 1 × 10⁵ CNE2 cells were seeded in each well of a six-well plate and cultured under proper condition. The cultured CNE2 cells were then used for plasmid transfection when cells covered 60% - 80%. Plasmids (5 µg) were transfected into CNE-2 cells with 12 µl LipofectamineTM 2000 (Invitrogen) following the manufacturer's protocol.

2.5. Reverse-Transcription PCR and qPCR

To evaluate the interference efficiency of constructed plasmids, the seven groups

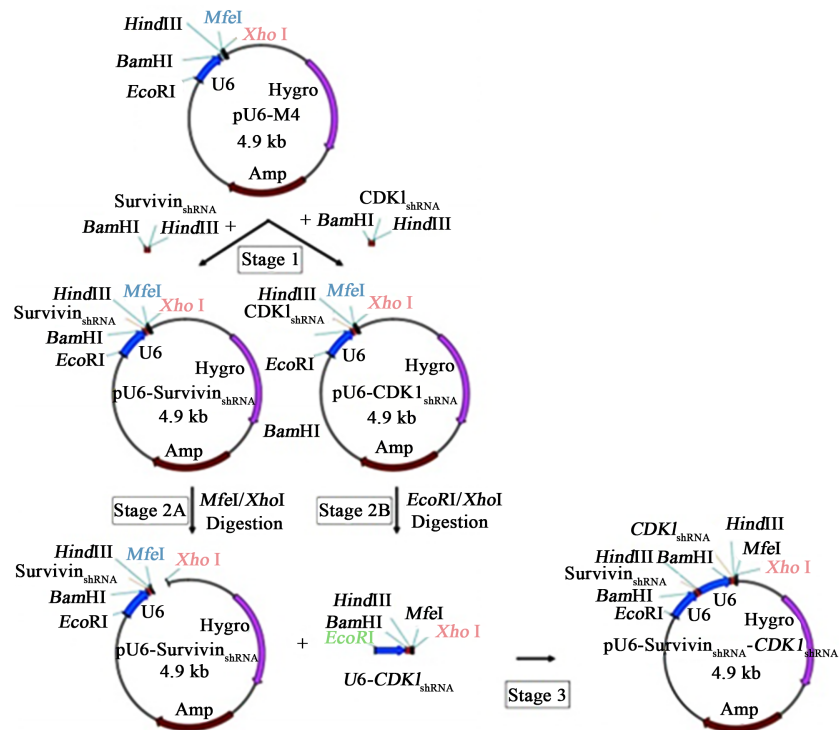


Figure 1. Three-step of constructing pU6-survivin_{shRNA}-CDK1_{shRNA}.

of CNE-2 cells were harvested after 48 h of transfection and total RNAs were extracted with RNA extraction kit (QIAGEN, Hilden, Germany). RNA was reverse transcribed by BioTakesupermo III RT Kit. qPCR was performed using the SYBR green Chimeric fluorescence method (Biotek Corporation, Beijing, China) following the manufacturer's Protocol. Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method with β -actin as internal reference and qualified with quantitative fluorescence PCR kit (Bio TeKe Corporation, Beijing, China). The 5' primer of survivin gene was 5'-TCAAGGACCACCGCATCTCTA-3', and the 3' primer was 5'-TGAAGCAGAAGAAACACTGGGC-3'. The 5' primer of CDK1 was 5'-CCTAGCATCCCATGTCAAAAACCTTGG-3' and the 3' primer was 5'-TGATTTCAGTGCCATTTTGCCAGA-3'. The 5' primer of β -actin that acted as internal reference was 5'-GTGGTGGTGAAGCTGTAGCC-3', and the 3' primer was 5'-GAGACCTTCAACACCC-3'. RT-PCR was performed with the following parameters: predegeneration at 95°C for 2 min, synthesis of cDNA with SuperScriptTM one-step RT-PCR kit on a PCR cyclor by heating 60°C for 1 min, followed by 40 cycles of amplification (denaturation at 95°C for 15 s, annealing at 55°C for 15 sec, extending at 72°C for 45 s), and a final extension step at 72°C for 5 min. The PCR cycle parameters were as following: predegeneration at 95°C for 30 sec; 40 cycles of amplification (denaturation at 95°C for 5 sec, and annealing at 60°C for 20 sec).

2.6. Western Blot

CNE-2 cells were harvested and washed with PBS, then lysed with 1 × SDS

loading buffer. Lysates from CNE-2 cells were added with protease inhibitor-cocktail (Roche) and 1 mM PMSF (Calbiochem). The supernatant was acquired with centrifuging at 13,000× g for 10 min. The lysates were denatured and separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to PVDF membranes (Millipore, Bedford, MA, USA). 5% nonfast milk in Tris buffered saline (TBS) buffer with 0.05% Tween-20 was used to block at room temperature for 1 hour. Then the primary rabbit antibodies of anti-human CDK1 (1:1000) and survivin (1:1000) were added and incubated at 4°C overnight. After five times of rinsing with TBST buffer, secondary goat anti-rabbit horseradish peroxidase conjugated antibody was added and incubated at room temperature for 2 h. Bands were visualized by ECL plus western blot detection reagent (GE, USA). The house-keeping gene β -actin was used as control and detected by using rabbit anti- β -actin antibody (1:4000, Abmart, China) plus HRP conjugated goat anti-rabbit secondary antibody (1:5000, Sigma).

2.7. Evaluation of Cell Growth Inhibition Rate (IR) by MTT Assay

CNE-2 cells were seeded into 96-well plates at a density of about 3×10^5 /well. CNE-2 cells were classed into five groups: transfection with pU6-survivin_{shRNA}; transfection with pU6-CDK1_{shRNA}; transfection with pU6-survivin_{shRNA}-CDK1_{shRNA}; transfection with pU6-survivin_{shRNA}-CDK1_{shRNA}; Control without adding anything. CNE-2 cells were transfected with 5 μ g plasmids and 5 μ l of Lipofectamine TM 2000 followed by incubating for 48 h. Then, 15 μ l of MTT (5 mg/ml) was added and cultured 4 h, followed by adding 150 μ l DMSO to each well. OD value of each well was analyzed at wavelength of 490 nm after shaking for 10 min, and the cell growth inhibition rate (IR) was calculated with the following formula: IR = (A control group – A experimental group)/A control group \times 100%. Each Experiment was repeated thrice.

2.8. Evaluation of CNE Cell Apoptosis by Flow Cytometry

The CNE-2 cells were categorized into the following five groups: transfection with pU6-survivin_{shRNA}; transfection with pU6-CDK1_{shRNA}; transfection with pU6-survivin_{shRNA}-CDK1_{shRNA}; transfection with pU6-survivin_{shRNA} NC-CDK1_{shRNA} NC; Mock transfection without adding anything. CNE-2 cells were harvested after 48 hour of post-transfection and digested with 0.25% trypsin to produce single-cell suspension, cells were adjusted to 1×10^6 /ml and then fixed with 70% cold ethanol at 4°C overnight. CNE-2 cells were further treated with Annexin V and PI and incubated for 30 min at room temperature in the darkness. The percentage of CNE-2 apoptosis was determined by Flow cytometry (Flow cytometry cycle detection kit, FACSort flow cytometry, B & D companies; USA) operated by a specialist.

2.9. Generation of Nasopharyngeal Carcinoma Xenografts and Evaluation of Antitumor Effects of RNA Interference

CNE-2 cells were trypsinized to produce suspension with DMEM medium, and

about 5×10^6 CNE-2 cells/200 μ l were injected subcutaneously into BALB/c mice to generate CNE-2 xenografts. Thirty BALB/c nude mice were divided randomly into five groups for experimental tests ($N = 6$): pU6-survivin_{shRNA}-CDK1_{shRNA} group (0.1 ml solution containing 50 μ g pU6-survivin_{shRNA}-CDK1_{shRNA}); pU6-survivin_{shRNA} (0.1 ml solution containing 50 μ g pU6-survivin_{shRNA}); pU6-CDK1_{shRNA} (0.1 ml solution containing 50 μ g pU6-CDK1_{shRNA}); pU6-survivin_{shRNA} NC-CDK1_{shRNA} NC (0.1 ml solution containing 50 μ g pU6-survivin_{shRNA} NC-CDK1_{shRNA} NC); mock transfection (0.1 ml PBS alone). Once tumors were palpable (mean diameter, 5 mm), PBS or various plasmids were injected into multi-position of tumor of mice. Tumor volume (V) was evaluated as following:

$$V = LW^2/2$$

(L: tumor length; W: tumor width). Tumor volume was evaluated every 3 days.

2.10. Statistical Analysis

Graphpad Prism 5 software was adopted for statistical analysis. Data were expressed as mean \pm SEM. One-way ANOVA with Tukey's test was applied for analysis data from multiple groups, while t test applied for data from two groups. The statistical significance was evaluated by $p < 0.05$ was considered statistically significant.

3. Results

3.1. pU6-Survivin_{shRNA}-CDK1_{shRNA} Was More Efficient than pU6-Survivin_{shRNA} and pU6-CDK1_{shRNA} in Knocking Down Survivin and CDK1 in CNE-2 Cells

Down-regulations of survivin and CDK1 were observed in CNE-2 cells interfered by pU6-survivin_{shRNA}, pU6-CDK1_{shRNA} and pU6-survivin_{shRNA}-CDK1_{shRNA}. Of the the designed sequences of shRNAs of survivin and CDK1, No. 3 of survivin and CDK1 manifested the highest efficiency of knocking down targeted gene, which was used for further construction of pU6-survivin_{shRNA}-CDK1_{shRNA} and subsequent experiments. CNE-2 cells interfered by pU6-survivin_{shRNA} (**Figure 2(a)**) or pU6-CDK1_{shRNA} (**Figure 2(b)**) displayed that the designed plasmids were effective in knocking down expressions of targeted genes.

pU6-survivin_{shRNA}-CDK1_{shRNA} were more efficient than pU6-survivin_{shRNA} and pU6-CDK1_{shRNA} in knocking down survivin and CDK1 in CNE-2 cells. The relative mRNA level of survivin in CEN-2 cells interfered by pU6-survivin_{shRNA}-CDK1_{shRNA} was about one third of that interfered by pU6-survivin_{shRNA} (0.18 vs 0.49, $p < 0.01$), and similar result observed in the relative mRNA level of CDK1 (0.15 vs 0.44, $p < 0.01$) (**Figure 2(c)**). Western blot analyses also supported pU6-survivin_{shRNA}-CDK1_{shRNA} was more efficient than pU6-survivin_{shRNA} and pU6-CDK1_{shRNA} in knocking down survivin and CDK1 in CNE-2 cells (**Figure 2(d)**).

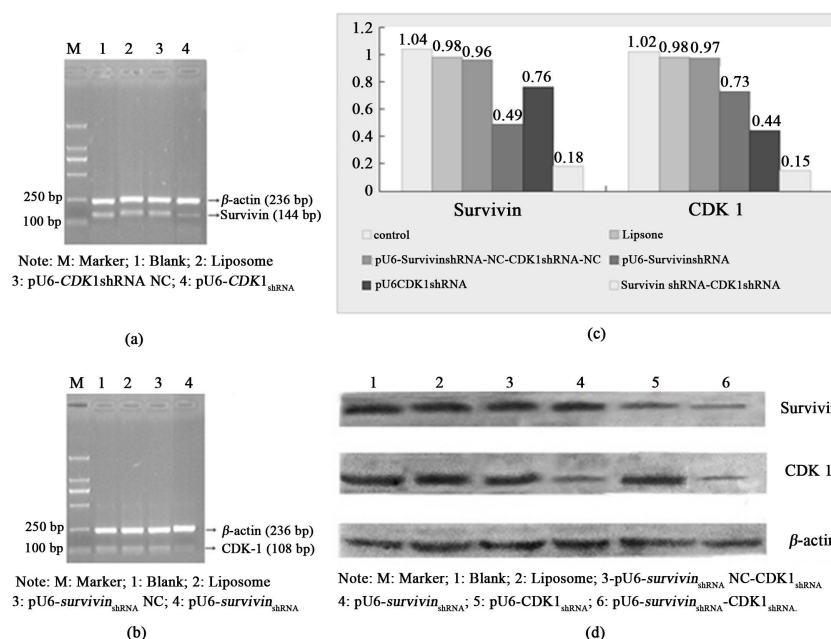


Figure 2. mRNA and protein levels of survivin and CDK1 in CNE-2 cells transfected with various recombinant plasmids. (a) RT-PCR analysis of survivin mRNA in CNE-2 cells transfected with pU6-survivin_{shRNA}; (b) RT-PCR analysis of CDK1 mRNA in CNE-2 cells transfected with pU6-CDK1_{shRNA}; (c) relative mRNA levels of survivin and CDK1 in CNE-2 cells transfected with different plasmids revealed by qPCR; (d) levels of survivin and CDK1 protein in CNE-2 cells transfected with different plasmids revealed by western blot.

3.2. pU6-Survivin_{shRNA}-CDK1_{shRNA} Were More Efficient than pU6-Survivin_{shRNA} and pU6-CDK1_{shRNA} in Inhibiting Growth and Inducing Apoptosis of CNE-2 Cells

The MTT colorimetric assay showed that CNE-2 cells transfected by pU6-survivin_{shRNA}, pU6-CDK1_{shRNA} and pU6-survivin_{shRNA}-CDK1_{shRNA} all resulted in growth inhibition. The rate of growth inhibition of CNE-2 cells transfected with pU6-survivin_{shRNA}-CDK1_{shRNA} was significantly higher than those transfected with both pU6-survivin_{shRNA} (41.21% vs 25.6%, $p < 0.01$) and pU6-CDK1_{shRNA} (41.21 vs 15.62%, $p < 0.01$) (**Table 1**). Flow cytometry also demonstrated that apoptosis in CNE-2 cells interfered by pU6-survivin_{shRNA}-CDK1_{shRNA} (15.2%) was higher than those interfered by pU6-survivin_{shRNA} (5.4%) and pU6-CDK1_{shRNA} (4.7%) (**Figure 3**).

3.3. The pU6-Survivin_{shRNA}-CDK1_{shRNA} Was More Effective in Inhibiting Tumor Growth in Nude Mice Received CNE-2 Xenograft

The tumor growth speeds of the pU6-survivin_{shRNA} NC-CDK1_{shRNA} NC group and mock transfection group were the fastest, and both were higher than those of the other three groups. As shown in **Figure 4**, the tumor volume of CNE-2 xenografts in nude mice treated with pU6-survivin_{shRNA}-CDK1_{shRNA} at a dose of 50 μ g three times for 3 weeks were significantly smaller than those of treated

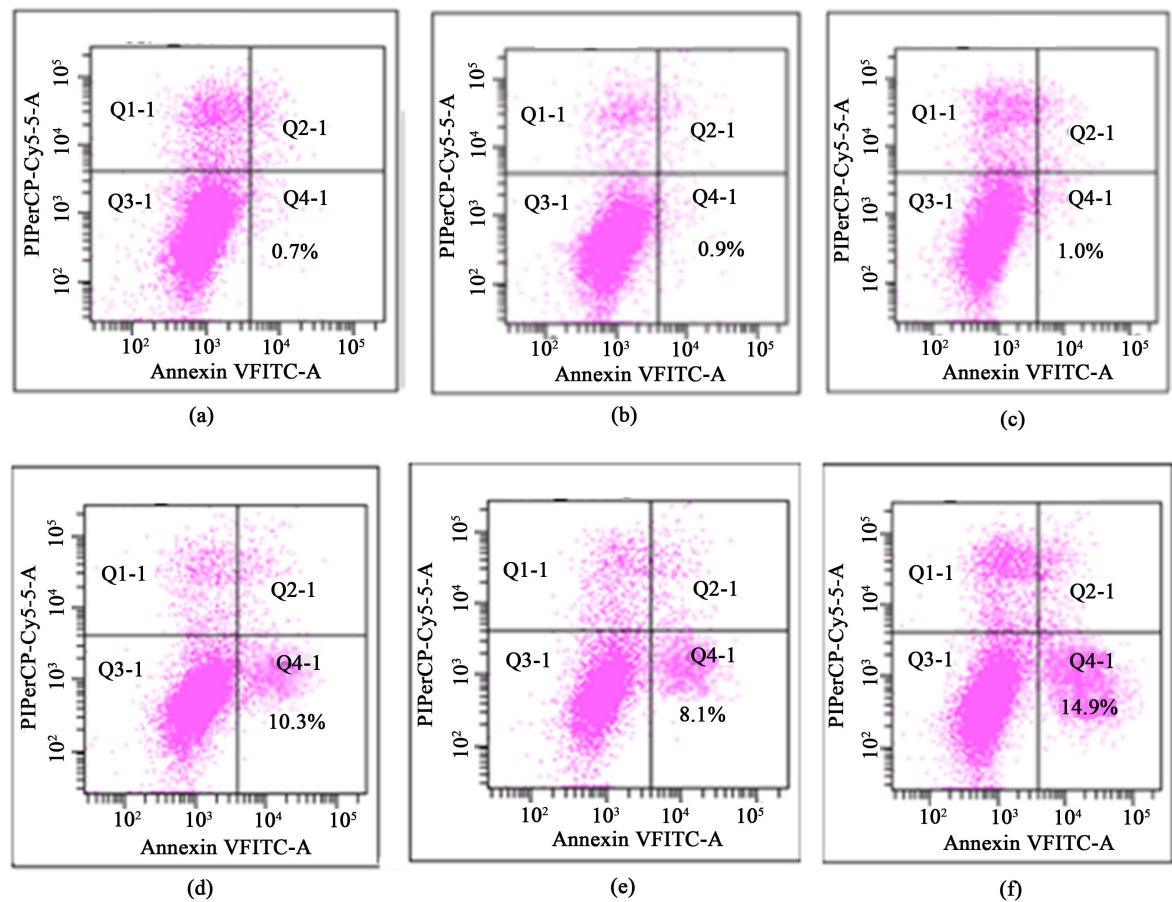


Figure 3. Apoptosis of CNE-2 cells treated with various components via FCS. Note. Q1: mechanical injured cells, Q2: necrotic cells, Q3: normal cells, Q4: apoptosis cells; (a) Blank control; (b) Liposome control; (c) pU6-Survivin_{shRNA}-NC-CDK1_{shRNA}-NC; (d) pU6-Survivin_{shRNA}; (e) pU6-CDK1_{shRNA}; (f) pU6-Survivin_{shRNA}-CDK1_{shRNA}.

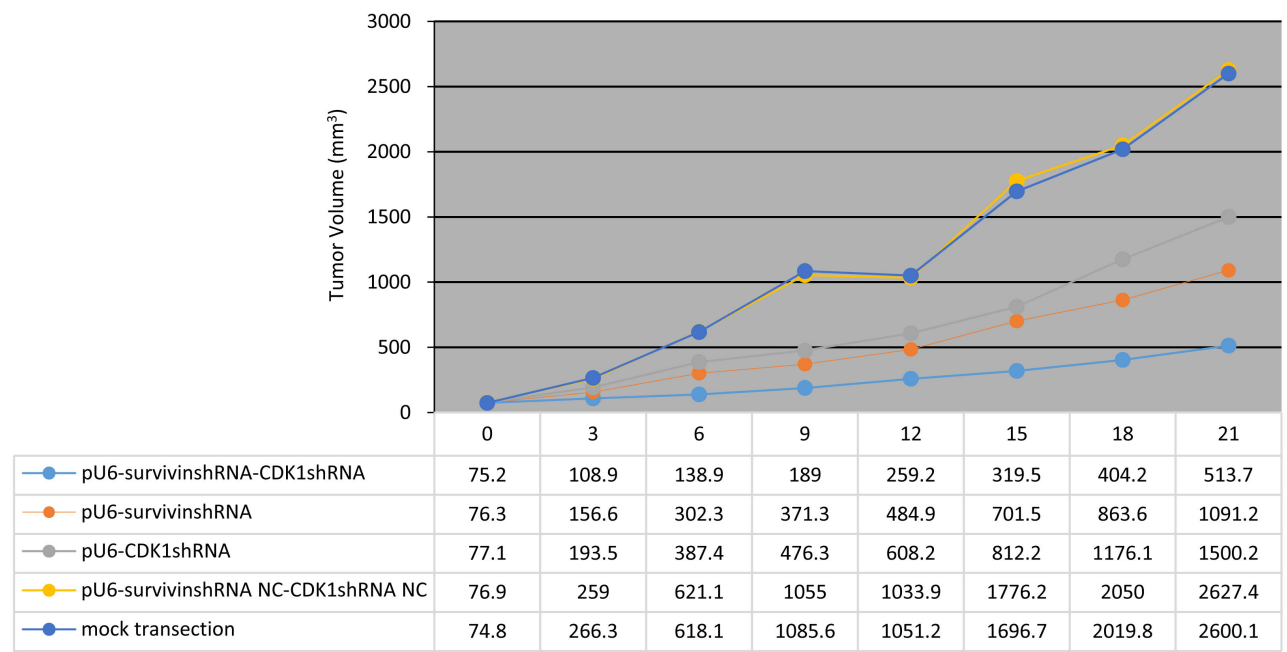


Figure 4. Growth curves of CNE-2 treated with different components.

Table 1. The growth inhibition rate of CNE-2 post-interference of 24 h.

	Opacity Density	Growth Inhibition (%)
control	0.461	
Liposome	0.46	0.22
pU6-Survivin _{shRNA} -NC-CDK1 _{shRNA} -NC	0.458	0.65
pU6-Survivin _{shRNA}	0.343	25.6
pU6-CDK1 _{shRNA}	0.389	15.62
Survivin _{shRNA} -CDK1 _{shRNA}	0.271	41.21

with pU6-survivin_{shRNA} (513.7 vs 1091.2, $p < 0.01$) and pU6-CDK1_{shRNA} (513.7 vs 1500.2, $p < 0.01$). Moreover, both pU6-survivin_{shRNA} and pU6-CDK1_{shRNA} manifested the activity of antitumor in mice received CNE-2 xenografts. No death, significant systemic adverse reactions and body mass changes were observed in mice during three weeks, and no obvious interclass differences observed in the volume of the tumors.

4. Discussions

Uncontrolled cell proliferation and abnormal cell death can lead to cancer. Therefore, therapies against cancer are to restore balance through targeting cancerous cells by blocking cellular growth or enhancing death. Expression of Survivin in cancer and their correlation with cell proliferation is well documented for various cancers, and silencing survivin has been proved to be efficient in treating cancers. Though knocking out survivin alone manifested antitumor effects both *in vitro* and *in vivo*, its efficiency didn't meet clinical demands, therefore, run on sentence some studies tried to combine silencing survivin and other treatments. In this study, we proved that the antitumor effects of targeting survivin and CDK1 perform significant better than either component alone.

In our nasopharyngeal carcinoma xenografts experiments, mice were administered with the three kinds of plasmids, *i.e.* pU6-survivin_{shRNA}-CDK1_{shRNA}, pU6-survivin_{shRNA} and pU6-CDK1_{shRNA}. They all manifested obvious inhibiting growth of CNE-2 xenografts. Meanwhile, pU6-survivin_{shRNA}-CDK1_{shRNA} was superior to pU6-survivin_{shRNA} and pU6-CDK1_{shRNA} in decreasing tumor volume, and the synergistic effects of targeting survivin and CDK1 were apparent (**Figure 3**). Our results also manifested that, compared with the single gene suppression, co-interfering survivin and CDK1 was more effective in reducing expressions of survivin and CDK1 in CNE-2 cells: levels of mRNAs and proteins of survivin and CDK1 in CEN-2 cells interfered by pU6-survivin_{shRNA}-CDK1_{shRNA} was significantly lower than those interfered by pU6-survivin_{shRNA} or pU6-CDK1_{shRNA} ($p < 0.01$) (**Figure 2(b)**, **Figure 2(c)**). The growth inhibition rate and apoptosis rate of CNE-2 cells were significantly increased suggested that survivin and CDK1 functioned synergistically. Moreover, the side effects of targeting CDK1 and survivin together co-building tandem shRNAs were not worse than unprofessional

either components alone. Taken together, our findings indicate that CDK1 and survivin act synergistically in antitumor activity.

Up-regulated expressions of survivin and CDK1 have been proved in various cancers. As a key nodal protein, survivin interacts with proteins of promoting mitosis (Cyclin D1, c-Myc and Stat3, e.g.), of preventing apoptosis (caspase3, Bcl-XL, e.g.) and of enhancing vascularization (VEGF) [2] [3] [4] [5] [6]. CDK1 is a key modulator involving the initiation and transition process in mitosis. CDK1 is also the enzyme for activating phosphorylation of multiples proteins, including survivin. Silencing CDK1 leads to inhibiting dephosphorylation of survivin (T34A) into Phosphorylation 34 site of survivin (T34E) [9]. It has been proved that interfering phosphorylation of survivin (T34A) will lower the level of survivin and promote apoptosis [14], which may be due to the incapability of survivin (T34A) combining with acceleration degradation of surviving by ubiquitin [15]. Therefore, silencing CDK1 and surviving simultaneously will generate synergistic effect in inhibiting growth and inducing apoptosis of tumor cells.

The combined targeting of survivin and CDK1 plasmids is superior to targeting of survivin and CDK1 alone *in vitro* and *in vivo*, the suppression of tumor growth was still incomplete. Many factors, including efficiency of transfection, expression levels and stability, result in incomplete inhibition of tumor growth. Moreover, no differential attack of constructed vectors, which shunts some plasmids to non-tumor tissue, also contributes to unsatisfactory antitumor effect. Therefore, it is necessary to solve the problem of designing efficacious *in vivo* delivery systems to transport designed vectors into tumor.

In brief, our results suggest that targeting survivin and CDK1 that based on constructed pU6-survivin_{shRNA}-CDK1_{shRNA} represents a more promising therapeutic approach for treating NPC than targeting survivin or CDK1 singly. The synergistic effects of silencing survivin and CDK1 suggest that targeting the two genes should produce robust efficacy against a variety of tumors.

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Conflicts of Interest

The authors declare no conflicts of interest.

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