Original Article
Lentivirus-mediated RNAi knockdown of HPV16E7 suppresses the proliferation of cervical cancer cell line CaSki in vitro and in vivo

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Abstract: Gene therapy is a relatively new method in medicine with large therapeutic potential. Until now, most clinical trials in gene therapy have focused on the treatment of cancer. Human papillomavirus type 16 E7 (HPV16E7) plays an important role in maintaining the malignant phenotype of cervical cancer cells. Hence, HPV16E7 becomes the suitable target for gene therapy in treating cervical cancer. In this research, we focus on inactivating HPV16E7 in CaSki cells and examine carcinogenicity of the cells. Lentivirus-mediated RNAi was used to down-regulate the expression of HPV16E7. RT-PCR and western blot were performed to detect the expression of HPV16E7. MTT, colony formation in vitro, tumor formation in vivo and apoptosis assay were used to detect the cell proliferation and apoptosis after down-regulation of HPV16E7. Flow cytometry was performed to analyze the changes in cell cycle in response to the down-regulation of HPV16E7. The delivery of lentivirus vector resulted in the decreased expression of HPV16E7 in the level of mRNA and protein. In addition, we found decreased proliferation and increased apoptotic rate in transfected CaSki cells. Furthermore, transfected CaSki cells were arrested in G0/G1 phase. In conclusion, HPV16E7 boosts proliferation of CaSki cells and contributes to tumorigenicity. Hence, HPV16E7 may be a pivotal gene target for therapeutic strategy in treatment of cervical cancer.

Keywords: HPV16E7, cervical carcinoma, RNA interference, lentivirus, proliferation

Introduction

Cervical cancer, the third most commonly diagnosed cancer, is the fourth leading cause of tumor death in females worldwide [1]. The development of cervical cancer is usually slow. Before carcinogenesis in cervix, cells generally go through some changes known as dysplasia. Then, the abnormal cells become cancer cells and invade into the cervix [2]. Symptoms may include vaginal bleeding, pelvic pain and pain during sexual intercourse. Hence, it is important to understand the cellular and molecular mechanisms of cervical cancer. There are many factors which determine the treatment procedure for cervical cancer, such as age, stage of cancer, and tumor type [3]. In present, women with early cervical cancer can be efficiently treated with radical surgery, and the addition of chemotherapy with fluorouracil and cisplatin to treatment with external-beam and intracavitary radiation can improve survival among women with locally advanced cervical cancer [4].

Human papillomavirus (HPV) is a risk factor for cervical cancer [5, 6]. It is reported that 93 percent of cervical cancer exists HPV. Human papillomavirus type 16 (HPV16), the most frequently type, is the most significant risk factor in its aetiology. HPV16 usually infects cell through microlesions of skin or mucosa. The infected cell divides and spreads laterally. Then the progeny migrate to the suprabasal differentiating cell layers when the viral genes such as E6, E7 are activated. With the duplication of DNA, viral particles form gradually which are released at the surface resulting in infection of other tissues.

HPV16E7 which is expressed by HPV16 plays an important role in malignant transformation. Compelling evidence suggests that HPV16E7 induces centriole amplification to promote the development of cancer resulting in aneuploidy of the E7-expressing cells [7]. In addition, HPV16E7 not only stimulates the S-phase genes cyclin A and cyclin E [8], but also blocks the function of the cyclin-dependent kinase inhibitors p21 and p27 which leading to tumorigenesis [9-11]. Furthermore, HPV16E7 has been reported to play an important role in the proliferation of various kinds of cells, such as thymic epithelium cell [12], keratinocytes [13] and laryngeal squamous carcinoma cell [14]. Hence the HPV16E7 may be a potential gene target for regulating the development of cervical cancer.

Gene therapy is a new technology in clinical trial that genes were delivered to or knocked down in the target tissues by vehicles [15]. Many cancers have been targeted throughout these years, such as skin, lung and cervical cancer. A number of different methods to cancer gene therapy are being developed, which mainly use viral vectors to invert anti-angiogenic factors, tumor-suppressor genes, prodrug-activating genes, immunostimulatory genes or interfere the expression of oncogenes [16]. The main viral vectors include oncoretrovirus, lentivirus, adenovirus, adeno-associated virus and herpes simplex-1 virus. In our research, as for its broad tropism and persistent gene transfer in most tissues, we chose lentivirus as viral vector to mediate the expression of HPV16E7 in cervical cancer. And more importantly, no one did this before.

Here, lentivirus-mediated RNAi was performed to inhibit the expression of HPV16E7. After knocking down HPV16E7, we detected cell proliferation, tumor formation, cell apoptosis and cell cycle phase distribution. Our results suggest that lentivirus-mediated RNAi knockdown of HPV16E7 suppresses the proliferation of cervical cancer cell line CaSki in vitro and in vivo.

Materials and methods

Cell line and culture

CaSki cells of human cervical cancer were cultured in RPMI 1640 media (Hyclone, USA) con-

taining 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 50 U/mL penicillin G and 50 U/mL streptomycin (Gibco) at 37°C under 5% CO_2 in humidified air. The cells were passed every 2-4 days.

Construction of lentivirus vectors

Short hairpin RNA (shRNA) targeting HPV16E7 sequence (GCT TCG GTT GTG CGT ACA A)and scrambled non-silencing RNA (TTC TCC GAA CGT GTC ACG T) were designed by using the manufacturer's RNAi Designer programme. DNA oligos containing the target sequence were chemically synthesized, annealed, and inserted into the expression vector by double digestion with Age Icand EcoR I, and ligation with T4 DNA ligase in accordance with the manufacturer's guidelines. The ligation was transformed into competent Escherichia coli DH5a cells. The correct transformant was identified by restriction enzyme analysis and DNA sequencing. The sequences were cloned into the pGCSIL-GFP lentiviral vectors. Expression vectors and package vectors were infected into 293T cells with the help of Lipofectamine 2000. Lentiviral particles such as pGCSIL-HPV16E7-shRNA-LV and pGCSIL-neg-shRNA-LV were harvested after 48 h. Lentiviral particles were purified using ultracentrifugation. The viral titer was determined.

Infection of lentivirus

CaSki cell suspensions were cultured in 96-well microplates with $5×10^3$ each well. For infection, cells were cultured in complete medium with lentiviruses (10 μg/mL) and treated for 24 h. Then, culture medium was substituted. Three groups of CaSki cells were used in subsequent assays: CON group (blank control group, with no infection), NC group (negative control group, infected with pGCSIL-neg-shRNA-LV), and KD group (HPV16E7 RNAi group, infected with pGCSIL-HPV16E7-shRNA-LV).

Western blot

Cell extracts were prepared in a lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0, 0.1% SDS, 1% sodium deoxycholate, 2 mM PMSF). Lysis buffer was prepared freshly, added to cells in six-well plates (100 μL/well) on ice, incubated for 10 min and centrifuged at 12,000 g in 4°C for 20 min to collect the super-
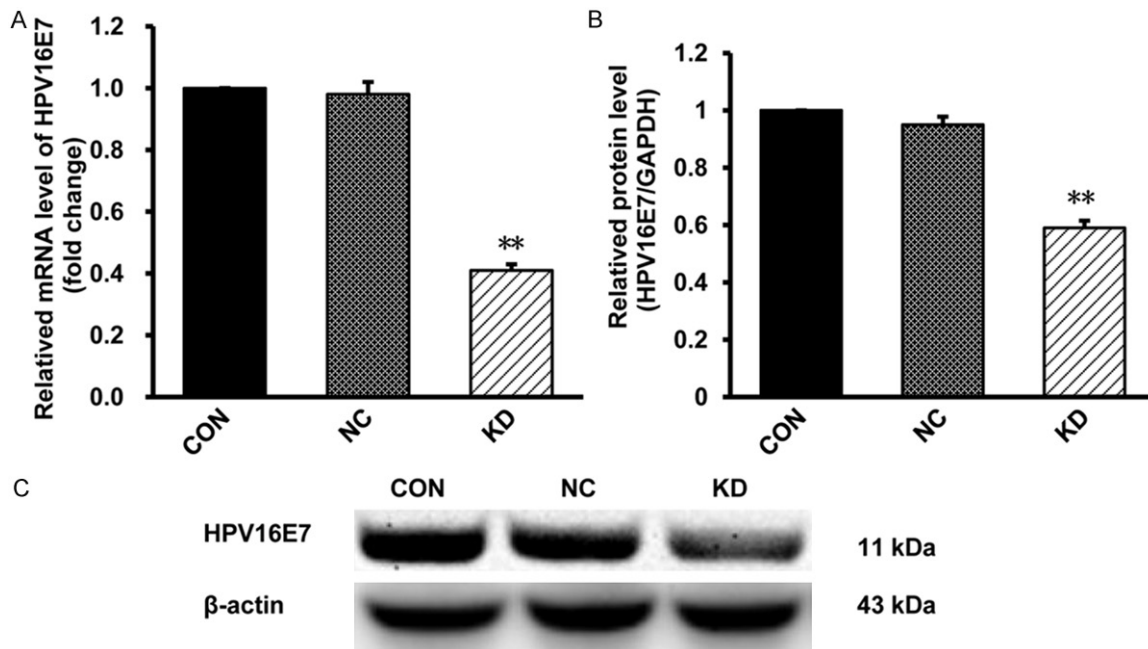
Figure 1. The HPV16E7 mRNA and protein expression following lentivirus-mediated RNAi knockdown. A. The mRNA level of HPV16E7 in different groups was assessed by qRT-PCR. The data are means ± SD, n=3, **P<0.01 vs the NC group. B. The HPV16E7 protein expression in different groups was detected by Western blot. C. The relative level of HPV16E7 to GAPDH. The data are means ± SD, n=3, **P<0.01 vs the NC group.

natant. After determining the protein concentration, protein samples were subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk for 2 h and incubated with primary antibodies against HPV16E7 and GAPDH at 4°C overnight. Then, the membrane was incubated with secondary antibody for 2 h. Before each step, membranes were washed by PBST for 3 times. The bands were visualized using an enhanced chemiluminescence system.

Real-time PCR

Total RNA was extracted from cells with Trizol reagent following the manufacturer's instructions. cDNA was obtained using M-MLV reverse transcriptase kit. Aliquots of cDNA were subjected to quantitative real-time PCR using Step One Plus Real-time PCR system. GAPDH mRNA expression level was used for normalization. The specific primer pairs were as follows: HPV16E7, sense: 5'-CGGGATCCATGCATGGAG-ATACA-3' and antisense: 5'-GCGGGCCCTTATG-GTTTCTGAGA-3'; GAPDH, sense: 5'-TGACTTCA-ACAGCGACACCCA-3'; antisense: 5'-CACCCTGT-TGCTGTAGCCAAAC-3'. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Proliferation assay

Lentivirus infected cells were cultured in 96-well plates for 24, 48, 72 h. The cells were incubated in the presence of 5 mg/mL 3-(4, 5-Di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Corp) for 4 h at 37°C. 100 µL of 20% SDS was added to each well. The absorption at 572 nm was measured using a spectrophotometer (Bio-Rad, USA).

In vitro colony formation assay

800 lentivirus-infected cells were seeded in 6-cm dishes and incubated for 14 days to form colonies. Then the cells were washed twice with PBS following fixation by 4% paraformaldehyde (PFA). Afterward, cells were stained with Giemsa for 20 min and washed twice by ddH$_2$O. The number of colonies (> 50 cell/colony) was counted manually.

In vivo tumor formation assay

All animal care and surgical procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Research
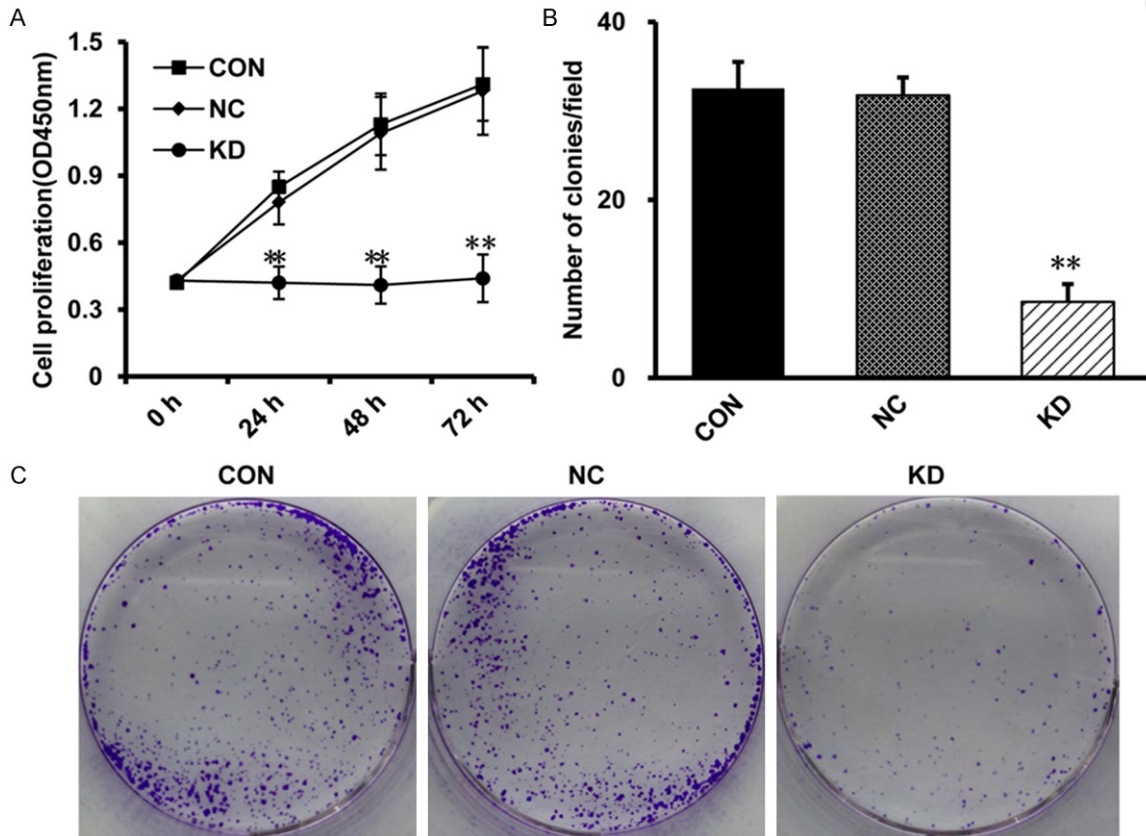
Figure 2. Inhibition of cell growth with HPV16E7 RNAi in CaSki cells. A. Cell proliferation was determined by MTT assay. Compared with the CON group and the NC group, cell proliferation in the KD group is suppressed at each time point (48 h and 72 h). The data are means ± SD, n=3, **P<0.01 vs the NC group. B. Colony formation assay was performed to comfirm the inhibitory effect of lentivirus-mediated RNAi. Compared with the CON group and the NC group, the clone number in the KD group was significantly decreased. The data are means ± SD, n=3, **P<0.01 vs the NC group. C. Colony formation of CaSki cells in vitro. CaSki cells in 6-cm dishes were cultured for 2 weeks and stained with Giemsa.

Council, 1996, USA). Five-week-old female immune-deficient nude mice (BALB/C-nu) were housed under a 12h light/dark cycle and the room temperature (RT) was kept at 26°C. Mice were randomly divided into CON group and KD group (n=3, each group). 2×10^6 CaSki cells were subcutaneously injected into the right flank of the mice with and without lentivirus-mediated RNAi knockdown of HPV16E7. After 4 weeks, xenografts were removed and weighed.

Apoptosis assay

A total of 1.0×10^6 CaSki cells were harvested by centrifugation and washed by PBS. Then, cells were incubated in binding buffer (1 mg/mL annexin V-APC) for 30 min. Fluorescence-activated cells sorting (FACS) analysis for

annexin V-APC staining was performed by flow cytometer (FACSCalibur™, BD, USA).

Cell cycle analysis

Lentivirus-infected cells were digested by trypsin and centrifuged at 1200 rpm for 5 min. Then cells were washed by ice-cold PBS and fixed by 70% ethanol. After discarding ethanol, cells were resuspended in 20 mg/mL propidium iodide (PI). Cells were analyzed by flow cytometer (FACSCalibur™, BD, USA).

Statistical analysis

All numerical data were described as means ± SD. Data were analyzed using the one-way ANOVA. A probability value of 0.05 or less was considered significant. Analyses were carried
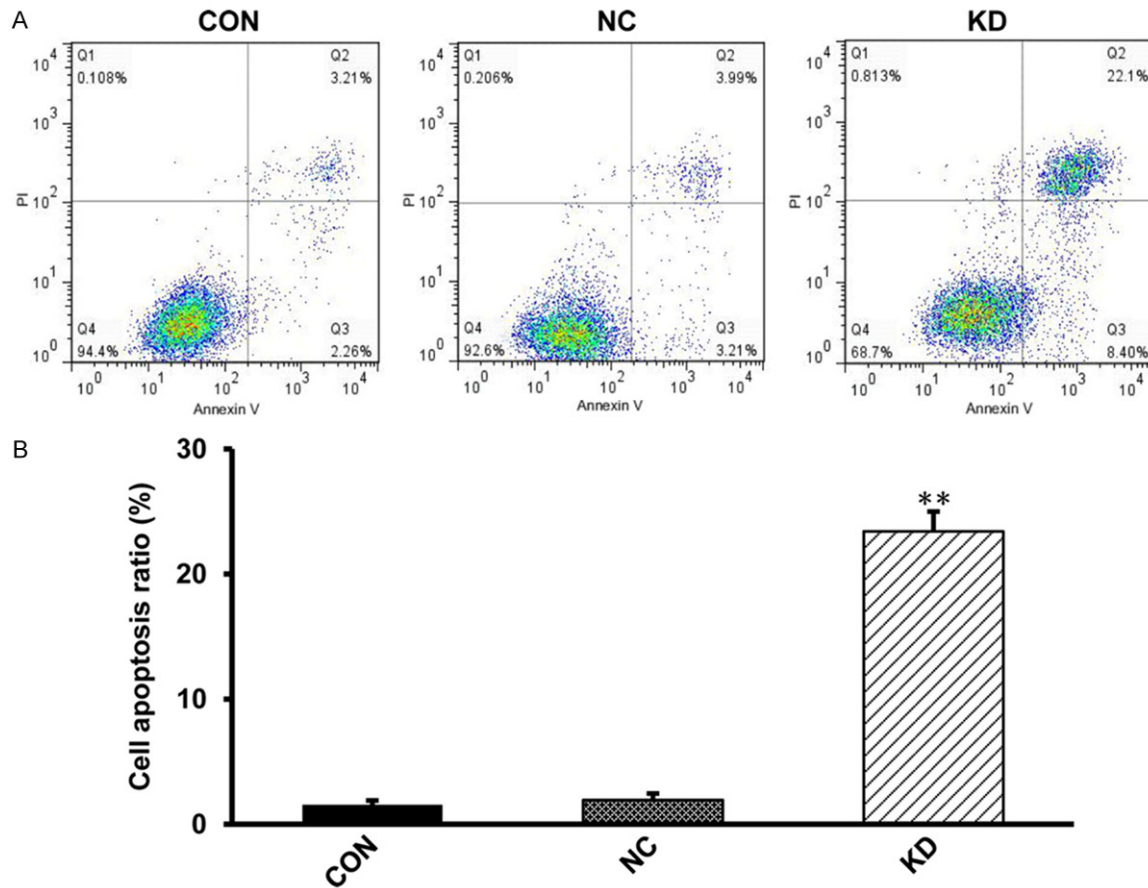
Figure 3. Apoptosis was detected by Annexin V-FITC. A. FCM data showed the apoptotic rate of CaSki cells infected with lentivirus was significantly increased. B. The cell apoptosis ratio in different groups. The data are means ± SD, n=3, **P<0.01 vs the NC group.

out using GRAPHPAD Prism Version 5.0 (Graph-Pad Software, La Jolla, CA, USA).

Results

HPV16E7-RNAi-LV down-regulates HPV16E7 expression in CaSki cells

After lentivirus-mediated RNAi knockdown of HPV16E7, the efficiency of HPV16E7-RNAi-LV was examined by RT-PCR and western blot. Compared with NC group, the expression of HPV16E7 mRNA and protein in KD group was significantly decreased, respectively (**Figure 1**). The mRNA level was reduced by 60.4%, while the protein level decreased by 40.8%. There is no significantly difference between CON and NC groups. These results showed that HPV16E7 was down-regulated efficiently in the lentivirus-infected CaSki cells.

Knockdown of HPV16E7 inhibits proliferation and clonogenicity of CaSki cells

To determine the role of HPV16E7 on cell proliferation, MTT was performed. The optical density (OD) of KD group was significantly lower than NC group at 48h and 72 h (**Figure 2A**) which suggested that CaSki cell proliferation was inhibited in KD group.

Then, colony formation assay was performed to confirm the effect of HPV16E7-RNAi-LV on ell proliferation. Compared with NC group, the number of colonies in KD group was decreased obviously (**Figure 2B**, **2C**) which demonstrated that HPV16E7-RNAi-LV resulted in suppression of CaSki cell proliferation. These findings declared that knocking down of HPV16E7 inhibited cell growth.
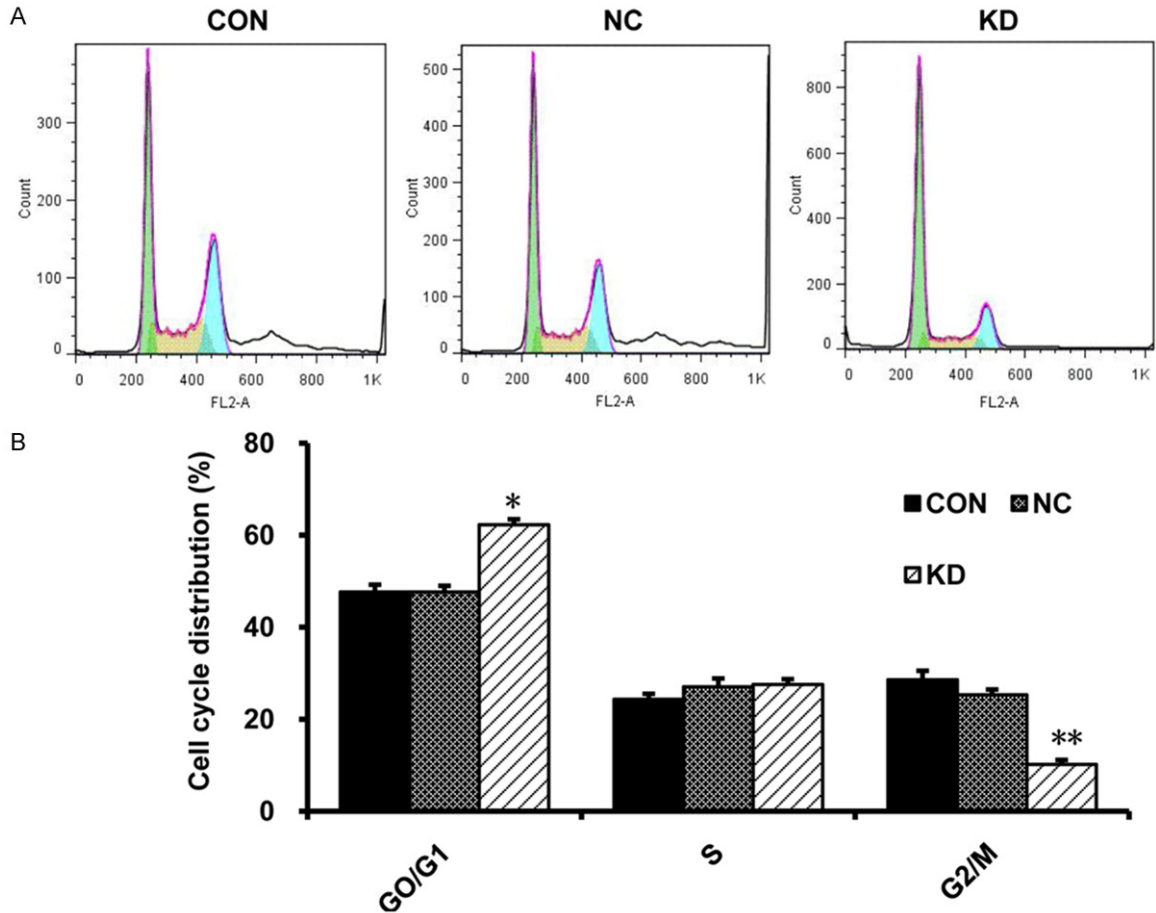
Figure 4. Cell cycle distribution in CaSki cells after lentivirus-mediated RNAi. A. FCM analysis suggested HPV16E7 RNAi arrests CaSki cells in the G0/G1 phase. B. Bar chart showed the distribution of cell cycle, the data are means ± SD, n=3, **P<0.01 vs the NC group.

Knockdown of HPV16E7 induces the apoptosis of CaSki cells

In order to examine the apoptosis of CaSki cells after knocking down HPV16E7, flow cytometry was performed. Apoptotic rate of the CON and the NC group are 1.53 ± 0.37% and 1.93 ± 0.52%, while it is 23.4 ± 2.6% in KD group. Hence the percentage of apoptosis of CaSki cells in KD group was increased compared with CON and NC groups (**Figure 3A**, **3B**). These data suggested that knockdown of HPV16E7 induced the apoptosis of CaSki cells which might be the cause of cell proliferation suppression.

Knockdown of HPV16E7 arrests CaSki cells in the G0/G1 phase

To investigate the cell cycle distribution after knocking down the expression of HPV16E7,

flow cytometry showed an increased frequency in the proportion of G0/G1 phase (62.27 ± 0.68% vs 47.64 ± 1.37%) but a decreased frequency in the proportion of G2/M phase (10.17 ± 0.18% vs 25.31 ± 2.15%) in KD group compared with NC groups (**Figure 4A**, **4B**). The results showed that knocking down HPV16E7 arrested CaSki cells in the G0/G1 phase.

Inhibition of HPV16E7 reduces tumor formation of CaSki cells in immune-deficient nude mice

To further conform the effect of HPV16E7-RNAi-LV on tumorigenesis, tumor formation assay was performed in vivo. Mice were injected with CaSki cells with or without lentivirus-mediated RNAi knockdown of HPV16E7, xenografts were removed and weighed 4 weeks later. In vivo tumor formation assay, all 3 mice of CON group
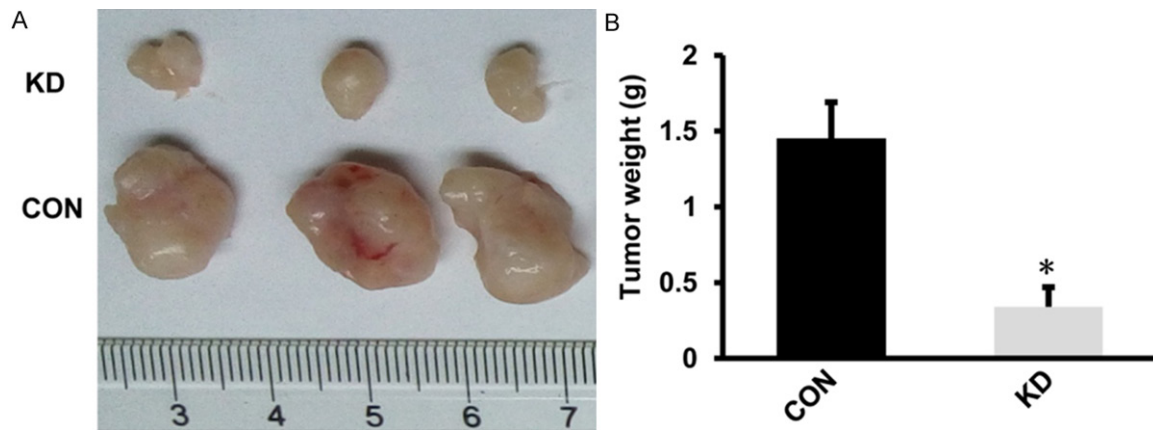
Figure 5. Tumor formation of CaSki cells in immune-deficient nude mice. A. Representative subcutaneous xenografts (arrows) produced by CaSki cells. B. Removed xenografts from CON group and KD group.

formed xenografts while the 3 mice of KD group formed smaller xenografts (**Figure 5A**). The weight of tumor in KD group was lower than those from CON group (**Figure 5B**). It demonstrated that inhibition of HPV16E7 reduces tumor formation of cervical cancer cells in vivo.

Discussion

The oncogenes in HPV16 are involved in cancer development and immortalization. Among these oncogenes in HPV16, HPV16E7 oncogene plays an important role in tumorigenesis. Varying degrees of regulation in a range of cellular and molecular processes were led by HPV16E7 through its interaction with other proteins [17] such as major histocompatibility complex-1 (MHC-1) [18-23], tumor suppressor protein pRb [24] and cell kinase dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) [25] which resulting in carcinogenesis. Furthermore, HPV16E7 has been reported to play an important role in the proliferation of various kinds of cells, especially cancer cells [14]. However, the role of HPV16E7-RNAi-LV in the inhibition of cell growth of cervical cancer has not been declared.

RNA interference (RNAi) is a widely used powerful tool in inducing loss-of-function phenotypes. The combination of lentivirus and RNAi is a new method to treat cancers and has been shown highly efficiency in treating variously cancers, according to several reports [26, 27]. In our study, we down-regulate the HPV16E7 gene in transfected cervical cell line CaSki cells by constructing an efficient and stable lentivirus vec-

tor. We decreased HPV16E7 expression in a cervical cancer cell line CaSki cells by lentivirus-mediated RNAi to study the effect of HPV16E7 silencing on the cell growth, apoptotic rate, cell cycle-progression in vitro and tumor formation in vivo.

Our results showed that after the delivery of lentivirus vector into CaSki cells, expression of HPV16E7 in the level of mRNA and protein were obviously decreased. Uncontrolled proliferation is one basic characteristic of tumor cells. In our study, HPV16E7 was knocked down by lentivirus in CaSki cells. MTT assay showed that knockdown of HPV16E7 significantly inhibited the viability of CaSki cells, and this inhibition was specific. Furthermore, this specific suppression was confirmed by the colony formation assay in vitro which indicated knockdown of HPV16E7 blocked colony formation in CaSki cells. In addition, silencing of HPV16E7 reduced the tumor formation of cervical cancer in vivo. Therefore, inhibition of HPV16E7 can suppress the proliferation of cervical cancer cells in vitro and in vivo.

The suppression of proliferation could be the result of apoptosis. Hence, we performed apoptosis assay. It demonstrated that the percentage of apoptotic rate in lentivirus-mediated RNAi knockdown of HPV16E7 cervical cancer cells was increased compared with cells without transfection.

Proliferation and apoptosis are related to cell cycle distribution. When cells arrested in G0/G1 phase, more apoptosis and less prolifera-

tion were observed. To explore the relationship among cell cycle distribution, proliferation and apoptosis, we used flow cytometry to detect cell cycle. In our research, HPV16E7-RNAi-LV arrested CaSki cells in G0/G1 phase. As for its effect on cell cycle distribution, more apoptosis and less proliferation were found in our study.

Previous research showed that the activation of HPV16E7 was able to boost cervical cancer cell growth which was regulated through interaction with other proteins, such as MHC-1 [28]. Moreover, HPV16E7 interacted with RB which resulted in blocking the activity of tumor suppressors [29]. Furthermore, HPV16E7 stimulated the S-phase genes cyclin A and cyclinE which contributed to tumorigenesis [8]. Hence, we speculate that HPV16E7 gene silencing may inhibit proliferation of cervical cancer.

HPV16E7 may be a potential therapeutic target in treatment of cervical cancer. Zheng et al [30] reported that a novel vaccine consisting of modified HPV16E7 fused with human cytotoxic T-lymphocyte antigen 4 (CTLA4) resulted in retarded tumor growth and prolonged survival in mouse injected with TC-1 cervical cancer cells in hind flank. Therefore, starting with HPV16E7 will open a new horizon for the treatment of cervical cancer.

In conclusion, we demonstrate that lentivirus-mediated RNAi knockdown of HPV16E7 can significantly repress cell proliferation in vitro and in vivo, induce apoptosis in CaSki cells and arrest cells in the G0/G1 phase. This provides a pivotal therapeutic strategy for treatment of cervical cancer.

Disclosure of conflict of interest

None.

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