Oxymatrine inhibits the proliferation of CaSki cells via downregulating *HPV16E7* expression

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Abstract. Treatment of recurrent and metastatic cervical cancer remains a challenge, especially in developing countries, which lack efficient screening programs. *HPV16E7* has been reported to play an important role in the development of cervical cancer. In recent years, oxymatrine, which was traditionally used as anti-malarial agent, has been shown to inhibit tumor growth with low toxicity to normal cells. In the present study, we investigated the mechanisms underlying the antitumor effect of oxymatrine in cervical cancer. The CCK-8 assay was used to compare the proliferation of untreated and oxymatrine-treated cervical cancer CaSki cells. Flow cytometry was applied to observe the effect of oxymatrine on apoptosis and the cell cycle distribution of CaSki cells. We used qRT-PCR and western blot analysis to determine the mRNA level and protein level of *HPV16E7*. The *HPV16E7* siRNA inhibition was also performed to confirm the effect of downregulating *HPV16E7* on the proliferation in CaSki cells. Our results revealed that oxymatrine-treated cells showed time-dependent and dose-dependent inhibition of proliferation and a significant increase in apoptosis. Oxymatrine arrested CaSki cells in G0/G1 phase and S phase while decreased the cells in G2/M phase. The expression of *HPV16E7* was significantly downregulated in oxymatrine-treated cells compared with control cells. Knock-down of *HPV16E7* effectively inhibited the proliferation of CaSki cells. In conclusion, our data suggest that oxymatrine inhibits cervical cancer growth via downregulation of *HPV16E7*. Oxymatrine can be considered to be a potential preventive and therapeutic target for cervical cancer.

Introduction

Cervical cancer, which accounts for 8% of all cancer deaths, is the third most common cancer in women after breast and colorectal cancer (1,2). The human papilloma virus (HPV) is known to be an essential cause of cervical cancer, among which 60% or more are with HPV type 16 (*HPV16*) (3). Carcinogenesis by *HPV16* is primarily attributed to the continuous expression of viral protein *E6/E7*. After infection of HPV, the integration of the viral *E6/E7* genes occur to the genome of the cervical epithelium, and the continued expression of *E6/E7* not only enhances the neoplastic progression to the cervical epithelium, but also drives the cervical cancer cells to malignant phenotype (4-6). There has been convincing evidence provided by previous studies that the overexpression of *HPV16E7* is associated with the development of cervical cancer (3,7). Therefore, the *HPV16E7* is considered to be potential therapeutic target for cervical cancer treatment.

Treatment strategies for cervical cancer today are focused on surgical operation or chemo-radiation. Although a number of chemotherapeutic drugs for treating cervical cancer can be used to control the growth of cancer and have shown certain therapeutic efficacy, the strong side-effects limit their application. Besides, only one-third of women with metastatic cervical cancer respond to chemotherapy and this response is short-lived (1,6). Therefore, to develop novel natural substances with curative selectivity for cervical cancer without showing significant toxic effect to normal cells is becoming much more necessary.

There has been a recent focus on the use of Chinese medicinal herbs to treat a number of diseases. Oxymatrine, which is a quinolizidine alkaloid extracted from the root of tradi-

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tional Chinese herbal medicine Sophora japonica (*Sophora flavescens* Ait), has been found to possess several biological effects such as anti-inflammation, inhibiting immune reaction, anti-virus and antitumor (8-13). Different from the traditional chemotherapy medicine, oxymatrine has been reported to inhibit the proliferation of a number of cancer cells *in vitro* and also inhibited viral-induced tumor formation in mice, with little influence to some normal cells (14,15).

As far as we know, there are few studies on the application of oxymatrine in the treatment of cervical cancer (10,16). Besides, we found no report concerning the putative relationship between oxymatrine and *HPV16E7* in anticancer study. In the present study, we investigated the effect of oxymatrine on cervical cancer cell line CaSki by evaluating cell proliferation, cell apoptosis, cell cycle, the mRNA and protein expression levels of *HPV16E7* gene *in vitro*. The aim of this study was to explore the mechanisms underlying the antitumor effect of oxymatrine on cervical cancer cells and support experimental data for the application of oxymatrine in the prevention and treatment of cervical cancer.

Materials and methods

Oxymatrine. We purchased oxymatrine from Chia Tai Tianqing Pharmaceutical Group Co., Ltd. (Nanjing, China). Before the application, we tested this oxymatrine and found that its purity is >99% indicated by SDS-PAGE analysis.

Cell line and culture. CaSki cells of human cervical carcinoma (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) penicillin (100 μg/ml) and streptomycin (100 U/ml) in 5% CO_2 atmosphere at 37˚C. The cells were passaged every 2-4 days to keep a proper density and cells in the logarithmic growth phase were used in the experiment.

Proliferation assay. CaSki cells in logarithmic growth phase were seeded in 96-well plates with $1x10^5$ cells each well, and cultured cells with RPMI-1640 complete medium. Twenty-four hours later, the medium was replaced with RPMI-1640 complete medium with various concentrations of oxymatrine (2, 4 and 6 mg/ml) and cultured continuously. In addition, control cells were incubated with medium only. After exposure to oxymatrine, the proliferation of CaSki cells was assessed by using Cell Counting kit-8 assay. After 24, 48 and 72 h, each well of cells were treated with 10 μl of Cell Counting kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) and incubated for 1 h at 37˚C. Spectrometric absorbance was measured at 450 nm by an enzyme immunoassay analyzer (Bio-Rad Laboratories, Hercules, CA, USA).

Colony formation assay. Colony formation assay was performed to observe the effect of oxymatrine on colony formation ability of CaSki cells. Cells ($1x10^3$) were plated in each well of 6-well plate and cultured with RPMI-1640 complete mediumin a humidified atmosphere with 5% CO_2 at 37˚C. After 2 weeks, the colonies were fixed by 4% paraformaldehyde (PFA) for 15 min and stained with Giemsa for 20 min. Cell aggregate consisting of 50 or more cells were

defined as one colony. Clonies were manually counted by microscope (Olympus, Tokyo, Japan) with x40 field.

Apoptosis assay. Apoptosis assay kit (Multi Sciences, Harbin, China) was used to treat the cell samples and the procedure followed the instructions of the kit. CaSki cells were treated with various concentrations of oxymatrine (0, 2, 4 and 6 mg/ml) for 72 h, and the cells were collected, washed, successively incubated with 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) for 15 min at room temperature. Before being subjected to flow cytometric analysis, apoptosic ratio of fixed cell sample was measured by flow cytometry (FACSCalibur™; BD Biosciences, San Jose, CA, USA).

Cell cycle analysis. CaSki cells were treated with different concentrations of oxymatrine (0, 2, 4 and 6 mg/ml) for 72 h. The cells were harvested by trypsinization, then were fixed with 70% ethanol and resuspended in 20 mg/Ml PI for 30 min. Flow cytometer (FACSCalibur™; BD Biosciences) was applied to detect DNA content. Based on the flow cytometry data, we determined the relative proportions of cells in the individual cell-cycle phase fraction.

Quantitative real-time PCR. Total RNA was extracted from CaSki cells which had been treated with 4 mg/ml of oxymatrine for 72 h or the control cells using TRIzol RNA reagent (Invitrogen, Carlsbad, CA, USA), and then was treated with DNAse I (Roche, Basel, Switzerland) to remove contaminating genomic and adenoviral DNA. MMLV reverse transcriptase kit (Promega, Madison, WI, USA) was used to convert the prepared total RNA to cDNA, which was then subjected to quantitative real-time PCR (qRT-PCR) using a SYBR-Green Master Mix kit on a Bio-Rad connect real-time PCR platform. Taq DNA polymerase was used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA levels. The sequences of primers were as follows: HPV16E7 (157 bp), sense: 5'-ATGCATGGAGATACACCT-3' and anti-sense: 5'-TTATGGTTTCTGAGAACA-3'; GAPDH (101 bp), sense: 5'-ACAACTTTGGTATCGTGGAAGG-3' and reverse, 5'-GCCATCACGCCACAGTTTC-3'. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. The protein was extracted from CaSki cells treated with 4 mg/ml of oxymatrine for 72 h and the control cells. The cells were lysed by lysis buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 and 50 mM Tris, pH 8.0), with the addition of 2 mM phenylmethylsulfonyl fluoride, and then centrifuged at 12,000 x g for 30 min at 4˚C. Protein concentrations were determined by protein assay kit (Sigma, St. Louis, MO, USA). Equal amounts of proteins (20 μg) were boiled for 10 min, and loaded onto a 15% SDS-PAGE gel and then transferred to the polyvinylidenedifluoride (PVDF) membranes. The membranes were incubated for 1 h with blocking buffer (PBS with 5% skim milk and 0.1% Tween-20) and then with anti-HPV16E7 primary antibody (Abcam, Cambridge, MA, USA) overnight at 4˚C. Secondary antibody (Abcam) was used to incubate the membranes for 1 h at room temperature. Before each step, membranes were washed 3 times by PBST. The protein bands were visualized by enhanced chemiluminescence. We used
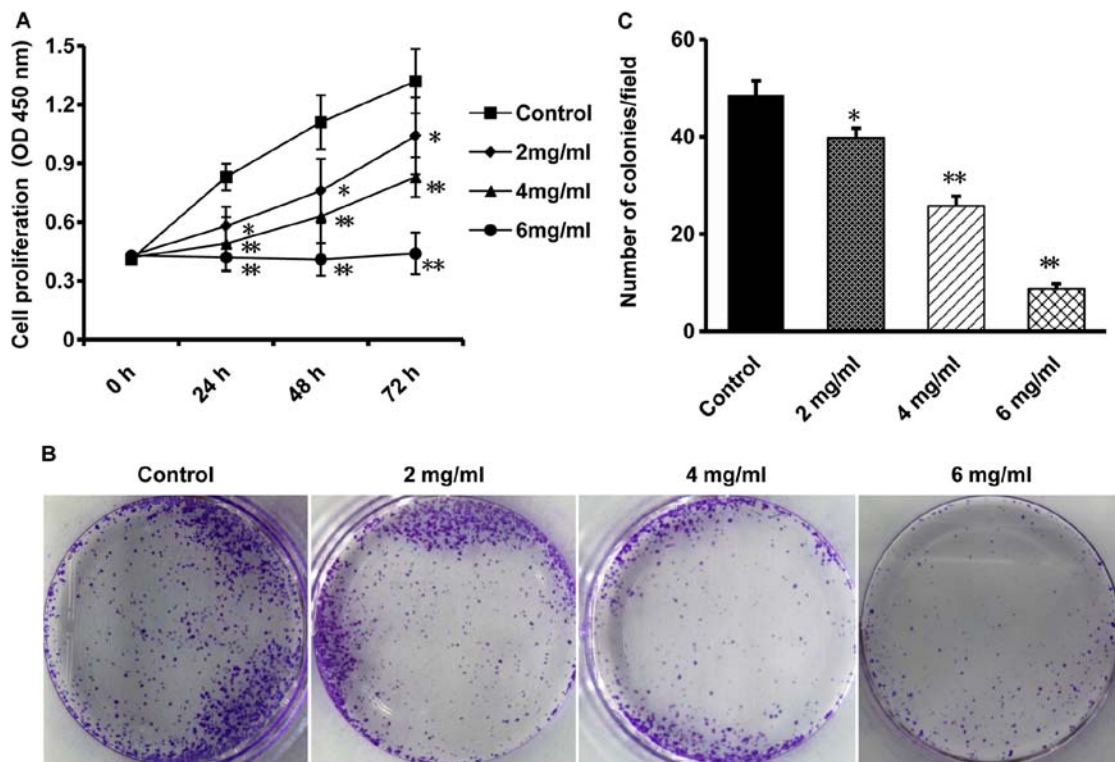
Figure 1. Oxymatrine inhibits the growth of CaSki cells. (A) CaSki cells were treated with oxymatrine at different concentrations (2, 4 and 6 mg/ml) seperately for various time. CCK-8 assay was used and the OD 450 nm values showed that oxymatrine could inhibit the proliferation of CaSki cells in both dose-dependent and time-dependent manner. (B) Representative images of colony forming assay for oxymatrine treatment group and the control group. (C) Colony formation assay results showed that the number of CaSki colonies in the oxymatrine group was obviously reduced, compared with the other groups and the suppressing effect was dose-dependent. *P<0.05 and **P<0.01, respectively, both indicating that a significant difference between the control group and the oxymatrine treated groups.

β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the internal control.

RNA interference. The siRNA for *HPV16E7* was designed and synthesized by Sangon Biotech Co., Ltd., (Shanghai, China), and the sequences were as follows (5'-3'): GCACACACGTA GACATTCGdTdT; (3'-5'): dTdTCGUGUGUGCAUCUGUA AGC. A scrambled siRNA which had no homology with human genome was also produced for use as a negative control. The siRNA duplex (50 nM) was transfected using Lipofectamine 2000 reagent (Invitrogen) following the instructions of the manufacturer. The CaSki cells were effected by *HPV16E7* siRNA for 72 h before the analysis of cell proliferation and *HPV16E7* expression.

Tumorigenicity assay. The ethics approval for this protocol was obtained from The Institutional Animal Care and Use Committee at Hubei University of Medicine. CaSki cells treated with 4 mg/ml of oxymatrine for 72 h and control CaSki cells were injected subcutaneously into 4-week old nude mice (3x10^6 cells/mouse), respectively. Four weeks after the injection, mice were sacrificed by injecting excessive chloral hydrate and tumors were separated and weighed.

Statistical analysis. All experiments were performed for at least three times, and data are shown as the mean ± SD. One-way ANOVA was used to determine statistically significant differences between groups, and P<0.05 was considered statistically

significant. Analyses were carried out using GraphPad Prism version 5.0 (Graphpad Software, Inc., La Jolla, CA, USA).

Results

Oxymatrine inhibits proliferation and clonogenicity of CaSki cells. CaSki cells were treated with various concentrations of oxymatrine (0, 2, 4 and 6 mg/ml) for 24, 48 and 72 h. CCK-8 assay was performed to determine the effect of oxymatrine on cell proliferation and found a time-dependent and concentration-dependent inhibition of oxymatrine to the growth of CaSki cells, compared with that in the control cells (P<0.05, n=6; Fig. 1A). Especially when the CaSki cells were treated with oxymatrine for 72 h, the inhibitory effect was much more significant than that treated for 24 h (P<0.05, n=6; Fig. 1A). Therefore, we chose the time-point of 72 h for further investigation.

The inhibitory effect of oxymatrine on CaSki cells was then confirmed by colony formation assay. The results showed that after treated with concentrations of oxymatrine (0, 2, 4 and 6 mg/ml), the colony formation ability of CaSki cells was significantly decreased in a dose-dependent manner, compared with the control group (P<0.05, n=6; Fig. 1B and C), indicating that oxymatrine is able to inhibit the growth of cervical cancer cells.

Oxymatrine induces apoptosis to CaSki cells. The effect of oxymatrine on cell death of CaSki cells was investigated by
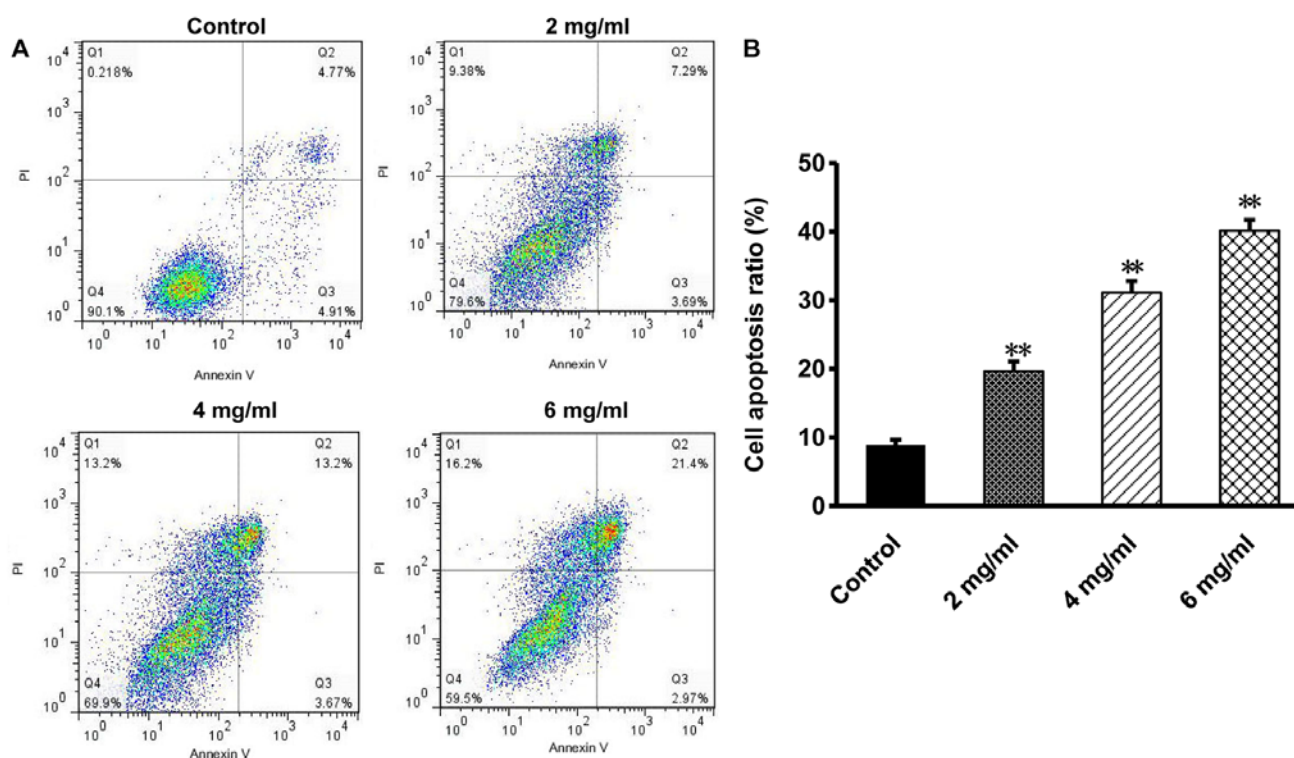
Figure 2. Oxymatrine induces apoptosis to CaSki cells. (A) Representative images of cell apoptosis analysis using Annexin V-FITC/PI with FCM for the oxymatrine treatment group and the control group. (B) Oxymatrine induced cell apoptosis of CaSki cells in a dose-dependent manner, compared with that in the control group. **P<0.01, indicating a significant difference between the control group and the oxymatrine treated groups.
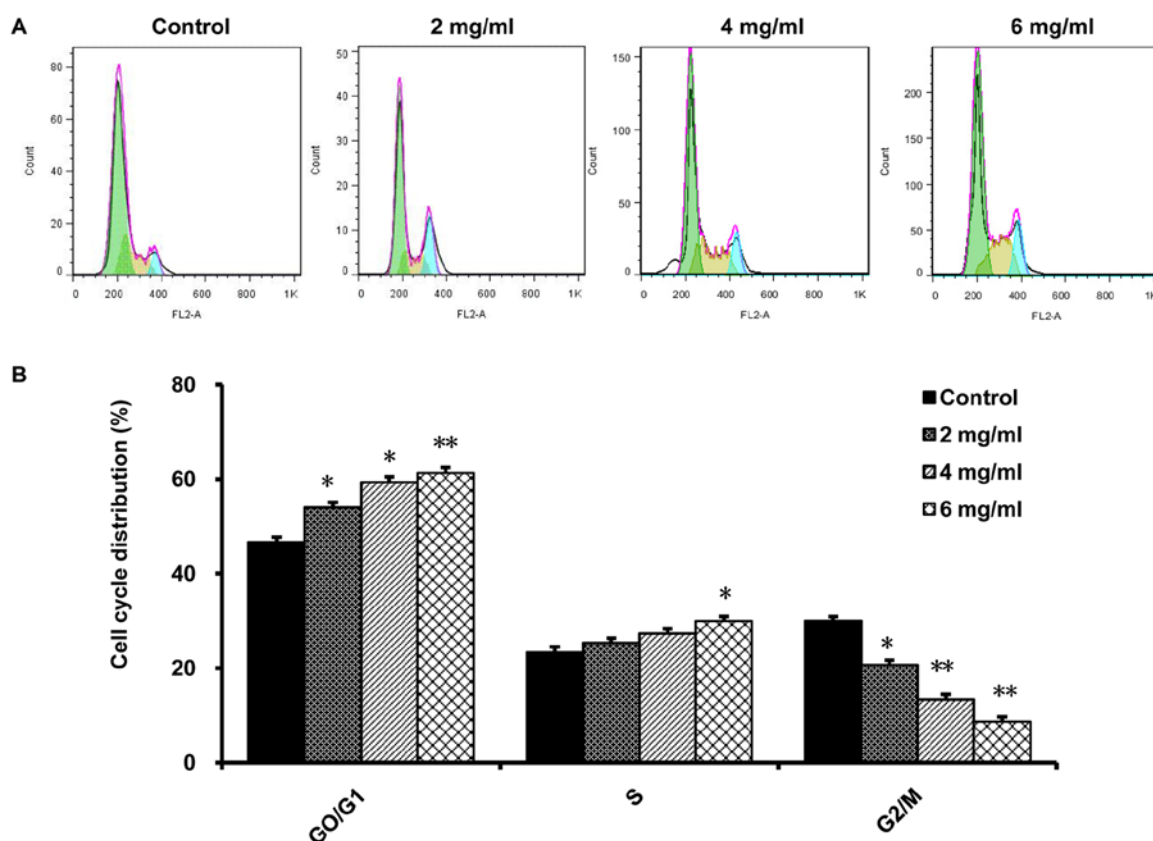

Figure 3. Oxymatrine changed the cell cycle of CaSki cells. (A) Representative images of cell cycle analysis using FCM for oxymatrine treatment group and the control group. (B) After treated with 2, 4 and 6 mg/ml oxymatrine, the proportion of CaSki cells in G0/G1 phase was prominently increased while the proportion in G2/M phase was decreased, compared with the control group. Besides, the proportion of CaSki cells in S phase was also significantly increased, in the 6-mg/ml oxymatrine treatment group. *P<0.05 and **P<0.01, respectively, both indicating a significant difference between the control group and the oxymatrine treated groups.
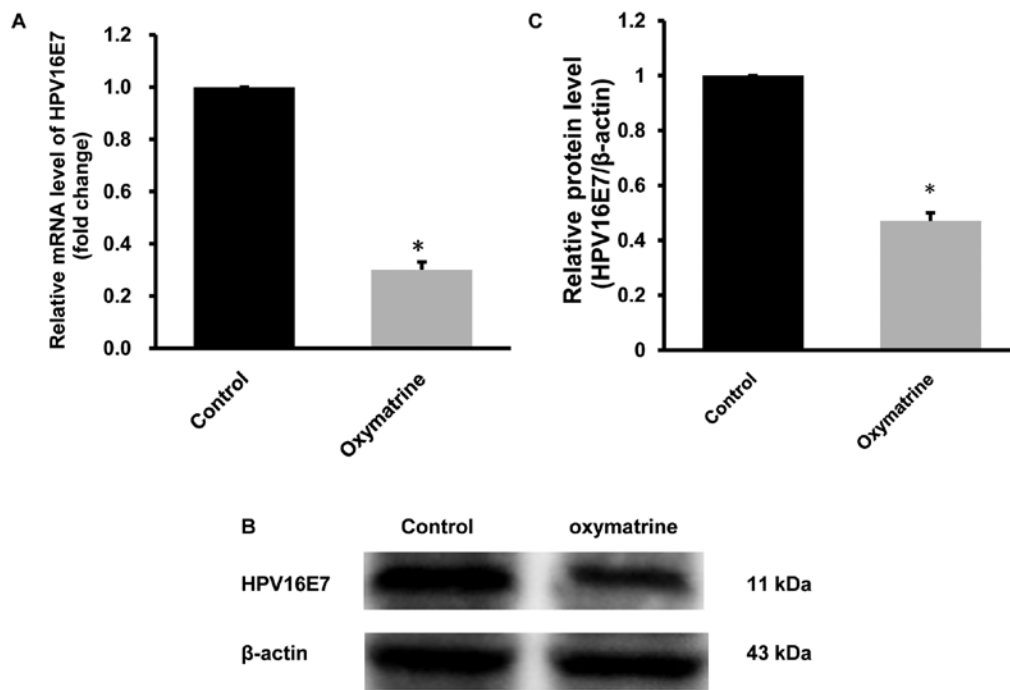
Figure 4. Oxymatrine downregulated the mRNA and protein level of HPV16E7 in CaSki cells. (A) The mRNA level of HPV16E7 was detected by qRT-PCR. The mRNA level of HPV16E7 was normalized to that of GAPDH. (B) Representative images of western blot protein gels for oxymatrine treatment group and the control group. (C) The protein level of HPV16E7 in the oxymatrine treatment group and the control group. **P<0.01, indicating a significant difference between the control group and the oxymatrine treated group.

evaluating the rate of apoptosis with flow cytometry. Results showed that after treatment with 2, 4 and 6 mg/ml of oxymatrine for 72 h, the rate of CaSki apoptosis was 19.62±1.71, 31.84±2.46 and 40.94±2.39%, respectively. The apoptosis rate of the control group was 9.64±0.98%. In contrast with the control group, the apoptosis rate of oxymatrine treated groups increased significantly in a dose-dependent manner (P<0.01, n=6; Fig. 2). As the results show, oxymatrine may suppress CaSki cell proliferation by causing cell death.

Oxymatrine changes the cell cycle distribution of CaSki cells. We performed FCM analysis to evaluate the effect of oxymatrine on the cell cycle of CaSki cells. After treatment with 2, 4 and 6 mg/ml of oxymatrine for 72 h, the proportion of G0/G1 phase was 54.91±2.08, 59.35±2.17 and 65.07±3.22%, respectively, while the percentage of G0/G1 phase control group was 44.35±1.78%. Compared with that in the control group, CaSki cells of the treated group in G0/G1 phase were markedly increased in a dose-dependent manner, while the cells in G2/M phase were decreased in the same way (P<0.01, n=6; Fig. 3). Besides, we found that 6 mg/ml oxymatrine could significantly increase the number of CaSki cells in S phase, compared to the control cells (P<0.01, n=6; Fig. 3). The results demonstrate that oxymatrine arrested CaSki cells in G0/G1 phase and S phase.

Oxymatrine downregulates the mRNA and protein levels of HPV16E7 in CaSki cells. We performed qRT-PCR and western blot analysis to examine the effect of oxymatrine on the expression of *HPV16E7* gene. The results showed that after treated with 4 mg/ml of oxymatrine, mRNA levels of *HPV16E7* gene in the CaSki cells was significantly decreased

by 64.5±2.8% (P<0.01, n=6; Fig. 4A) and protein level was prominently decreased by 52.3±3.2% (P<0.01, n=6; Fig. 4B and C), compared with the control group. These data demonstrate that oxymatrine could markedly downregulate the expression of *HPV16E7* in CaSki cells.

HPV16E7 siRNA suppresses the proliferation of CaSki cells. We used *HPV16E7* siRNA to knock down *HPV16E7* mRNA level to investigate the effect of downregulating *HPV16E7* on the proliferation of CaSki cells. Our results show that after treated with *HPV16E7* siRNA, the mRNA and protein level of *HPV16E7* in CaSki were reduced by 66.4±2.7 and 57.2±6.1% respectively, in contrast with the control group treated with scramble siRNA (Fig. 5A-C). After treated with *HPV16E7* siRNA, the proliferation of CaSki cells was suppressed in a time-dependent manner, compared with control siRNA (P<0.05, n=6; Fig. 5D). Our data demonstrate that knocking down *HPV16E7* expression could suppress the proliferation of CaSki cells.

Oxymatrine suppressed the growth of CaSki cells in vivo. CaSki cells (3x10^6) treated with oxymatrine or control cells were injected subcutaneously into each athymic nude mouse. Four weeks after the injection, mice were sacrificed and tumors were separated. As showed in the tumor images, all 3 mice injected with control CaSki cells formed xenografts. In contrast, 2 out of 3 mice injected with oxymatrine-treated CaSki cells formed smaller xenografts. The weight of tumors from oxymatrine treated CaSki cells was significantly lower than those from the control CaSki cells (P<0.05, n=3; Fig. 6B). The results suggest that oxymatrine could suppress tumorigenicity of cervical cancer cells *in vivo*.
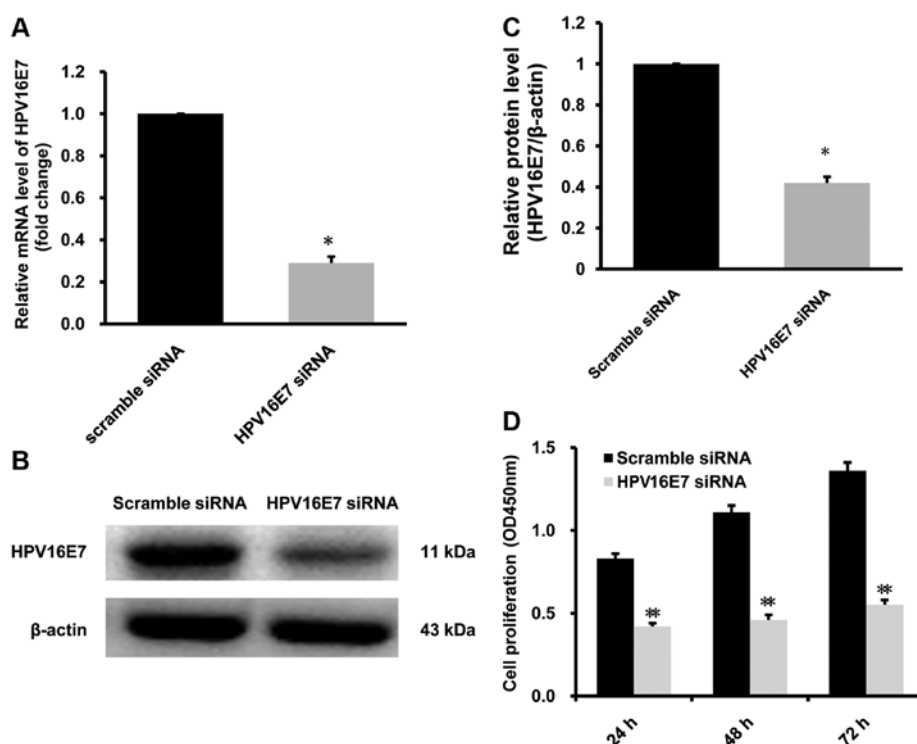
Figure 5. HPV16E7 siRNA downregulates the expression of HPV16E7 and suppresses proliferation in CaSki cells. (A) The mRNA level of HPV16E7 was detected by qRT-PCR. mRNA expression of HPV16E7 was normalized to that of GAPDH. (B) Representative images of western blot protein gels for HPV16E7 siRNA treatment group and the scramble control group. (C) The protein level of HPV16E7 in the HPV16E7 siRNA treatment group and the scramble control group. (D) CCK-8 assay showed when treated with HPV16E7 siRNA for 24 and 48 h, the proliferation of CaSki cells was significantly suppressed, compared with the scramble control group. **P<0.01, indicating a significant difference between the scramble control group and the HPV16E7 siRNA group.
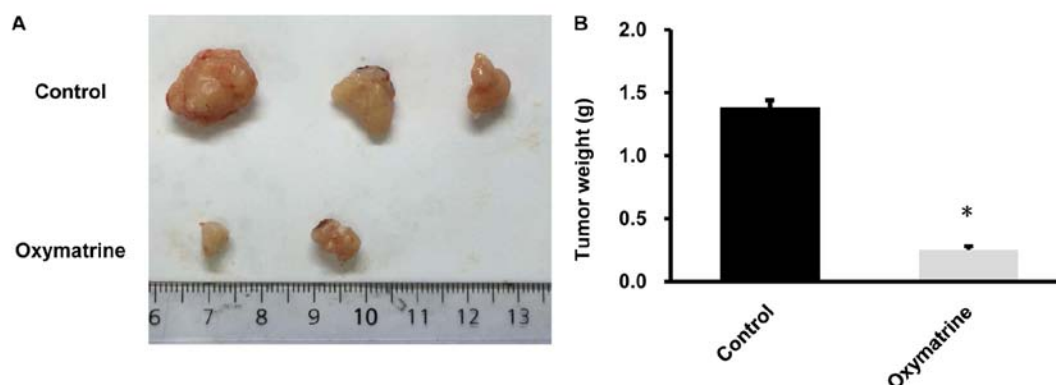

Figure 6. Oxymatrine inhibits the proliferatin of CaSki cells *in vivo*. (A) The separated tumors derived from oxymatrine treated CaSki cells and the control cells. (B) The weight of tumors of oxymatrine treatment group was significantly lower than that of the control group. *P<0.05, indicating a significant difference between the control and the oxymatrine treated group.

Discussion

Oxymatrine is a major alkaloid component found in the roots of Sophora species (17). It is commonly used for the treatment of liver disorders and other diseases such as arrhythmia, eczema and skin disorders, leukopenia and bronchitis (13,18-21). Some studies have also reported oxymatrine showed anticancer activity in human gastric cancer cells, pancreatic, ovarian cancer, and human breast cancer cells (14,16,22). However, to the best of our knowledge, the mechanisms of the antitumor effect of oxymatrine on cervical cancer have not yet been elucidated. In the present study, various concentrations of oxymatrine were used to treat cervical cancer cell line CaSki cells, and we found that oxymatrine treatment could induce inhibition of proliferation and apoptosis in cervical cancer CaSki cells *in vitro* and *in vivo*.

One of the main reasons for tumorigenesis is loss of control of cell proliferation, which is involved both in tumor initiation and progression (23). Therefore, we applied CCK-8 assay and found that oxymatrine could obviously inhibit cell proliferation in cervical cancer CaSki cells in a time-dependent and dose-dependent manner *in vitro*. Besides, our colony formation assay results also showed similar inhibition effect of oxymatrine on the colony forming ability of CaSki cells in

a dose-dependent manner. *In vivo*, we performed analysis of tumorigenesis and found that the weight of tumors resulted from CaSki cells treated with oxymatrine were significantly reduced, compared with the tumors derived from control CaSki cells. These data indicate that oxymatrine can suppress the growth of cervical cancer cells *in vitro* and *in vivo*.

Apoptosis is the process of cell death, with a series of cellular and molecular changes, such as phosphatidylserine externalization, chromatin condensation and cell shrinkage. Apoptosis plays important functions in organ development, homeostasis and immune defense. Uncontrolled cell death is another main reason for tumorigenesis (24,25). Therefore, inducing cell apoptosis of tumor cells is always of great importance in developing anticancer treating methods. In the present study, flow cytometry was applied to the effect of oxymatrine on the apotosis of CaSki cells and the results demonstrated that CaSki cells could be induced to apotosis by oxymatrine in a dose-dependent manner.

Since the change of cell proliferation and apotosis is intimately correlated with the change of the cell cycle (23,24). In this study, in order to investigate the cell cycle related mechanism of the effect of oxymatrine on the proliferation and apotosis on CaSki cells, we also performed flow cytometry and found that the oxymatrine treated CaSki cells were significantly blocked in G0/G1 phase. After treated with increasing concentration of oxymatrine, CaSki cells in G0/G1 phase were prominently increased while cells in the G2/M phase were obviously decreased, in contrast with control CaSki cells. Moreover, we found that oxymatrine could increase the number of CaSki cells in S phase to a certain extent, and 6 mg/ml made a significant difference compared to the control cells. When arrested in G0/G1 and S phase, the CaSki cells tend to decrease the proliferation rate with more apoptosis, which is also in accordance with our proliferation and apoptosis assay data. These data suggest that the growth inhibition and apoptosis induction of CaSki cells may result from the effect of oxymatrine on arresting CaSki cells in G0/G1 phase.

According to previous studies, over 60% of all cervical cancers are closely correlated with *HPV16*, which encodes the essential genes for virus replication, *E6* and *E7* oncogenes (7). The viral protein *E7* can cause destabilization and the disruption of *Rb/E2F* complexes, which is essential for driving the process of cell cycle into S phase, and upregulating the anti-apoptotic protein *Bcl-2* to promote cell survival and proliferation (3,26). Several researches have reported that inhibition of the expression of *E7* oncogene was efficient in HPV-associated cancer therapy (15,27,28). It is reported by previous studies that oxymatrine showed anti-hepatitis virus effects (29-31). Although no report on the anti-HPV effect was found, our results demonstrated that oxymatrine could decrease both the mRNA and protein levels of *HPV16E7*. Since the expression of *HPV16E7* was closely correlated with the proliferation and apoptosis in CaSki cells, we speculated that oxymatrine could inhibit proliferation and enhance apoptosis in CaSki cells via downregulating *HPV16E7*. We also used *HPV16E7* siRNA to knock-down *HPV16E7* expression, and the results indicated that downregulating *HPV16E7* expression could suppress the growth of CaSki cells, which was in accordance with our speculation. We also hypothesized that oxymatrine is able to treat cervical cancer through the anti-*HPV* effect, which is going to be the subject of our further investigation.

In conclusion, our data showed that oxymatrine significantly induced cell apoptosis, and suppressed cell growth of cervical cancer CaSki cells both *in vitro* and *in vivo*. Oxymatrine could decrease the CaSki cells in G2/M phase and arrest the CaSki cells in G0/G1 and S phase, which partly mediated proliferation inhibition and apoptosis induction. Besides, we a found that oxymatrine could downregulate the expression of *HPV16E7* at the mRNA and protein level, which offered the molecular explanation to the cell growth inhibitory effect of oxymatrine on CaSki cells. Therefore, we believe that oxymatrine can be developed to a potential preventive and therapeutic candidate for cervical cancer treatment. Our data may provide theoretical support for the clinical anticancer application with oxymatrine. However, the toxicity of oxymatrine and its effect on the proliferation of normal cells were not assessed in the present study. In future, we will continue our study on the toxicity of oxymatrine and other effects of oxymatrine in carcinoma cells *in vivo* and *in vitro*.

Acknowledgements

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