

# HPV16 E6-178G/E7-647G Promotes Proliferation and Inhibits Apoptosis in Cervical Cancer C33A Cells

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## Research article

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# Abstract

**Background** HPV16 is the main cause of cervical cancer. In our study, we aimed to investigate the role of HPV mutants HPV16 E6-178G/E7-647G in the proliferation and apoptosis of cervical cancer C33A cells.

**Methods** Plasmids encoding the HPV16 E7 prototype (E7-647A)-GV144, E7 mutant (E7-647G)-GV144, HPV16 E6/E7 prototype (E6-178T/E7-647A)-GV144, and E6/E7 mutant (E6-178G/E7-647G)-GV144 were stably transfected into cervical cancer C33A cells. Western blot analysis, CCK8 proliferation assay, cell cloning assay and flow cytometry were used to detect the effects of the different polymorphism sites in HPV16 on cell proliferation and apoptosis.

**Results** HPV16 mutations promoted the proliferation and inhibited the apoptosis of cervical cancer C33A cells, and the effect of the E6-178G/E7-647G co-mutation was significantly greater than that of the single E7-647G mutant ( $P<0.05$ ).

**Conclusions** HPV16 E6-178G/E7-647G can thus promote the proliferation and inhibit the apoptosis of cervical cancer cells.

## Highlights

1. In the present study, we found that the rate of the *E7* gene A647G mutation in cervical cancer tissue was higher than in non-cervical cancer tissue
2. HPV16 E7-A647G and E6-T178G co-mutations have been demonstrated in our previous studies
3. HPV16 E6-178G/E7-647G can thus promote the proliferation and inhibit the apoptosis of cervical cancer cells.

## Background

Cervical cancer is one of the most common malignancies in women and the fourth leading cause of cancer death in women, according to GLOBOCAN's latest estimate, there will be 604,000 new cases of cervical cancer and 342,000 deaths from this disease worldwide in 2020[1]. Human papillomavirus (HPV) vaccination programs have been successful in preventing cervical cancer in some developed countries, but global coverage remains low [2, 3]. The incidence and mortality rates of cervical cancer in China are 7.5 and 3.4 per 100,000 women, respectively [4, 5]. Therefore, cervical cancer remains an important public health issue in China.

Currently, more than 200 gene sequences have been identified from HPV genotype-specific sequence information. According to the transformation characteristics of HPV, 14 types, including HPV-16, -18 and -31, were classified as high-risk by WHO [6–8]. Epidemiological data has confirmed[9] that persistent infection by high-risk HPV can lead to cervical atypical hyperplasia and cancer through the *E6* and *E7*

genes, which are the two major cancer genes which target a variety of tumor suppressor proteins including p53 and pRb.

Evolutionary analysis has shown that globally, the diversity of the HPV16 genome has been evolving for more than 200,000 years [10]. Epidemiological studies have shown that mutations in the HPV16 gene may contribute to persistent viral infection and the development of cervical cancer. For example, Villa et al [11] showed that non-European variants have a general tendency to lead to persistent infection and are associated with cervical lesions. Zhang et al.[12] found that the European variant containing the T350G mutation results in the replacement of valine by leucine in the E6 protein, which is considered to be an additional risk factor for persistent infection and cervical lesions. In addition, the prevalence of polymorphic cervical cancer with *E6* T178G mutations was much higher in Asia (65.5% in China, 85.2% in South Korea and 44% in Japan) than in Europe (2%) and North America (3%). However, little is known about the carcinogenic potential of the HPV16 variant in Asian women compared to many studies in European and American populations [13]. Because of the *E6* gene encodes the major transforming protein that inhibits cell apoptosis and promotes cell proliferation, and it is associated with cancer invasiveness, most studies of HPV16 variations have focused on the *E6* gene [14], while relatively few studies have analyzed gene variations in *E7* as the other major oncogene of HPV16. Studies have shown [15] that HPV16 E7 induces upregulation of KDM2A, inhibits mir-132 and promotes proliferation of cervical cancer cells, thus leading to malignant progression of cervical cancer that is related to poor prognosis for cervical cancer patients. Based on the above, the main purpose of our study was to observe whether the HPV16 mutant would change the carcinogenicity of the virus.

## Materials And Methods

### Tissue samples

Approved by the Medical Ethics Committee of the First Affiliated Hospital of Shihezi University Medical School (Approval Number: 2019-017-01), female patients who were treated at the Friendship Hospital and the People's Hospital of Kashgar who were diagnosed with cervical lesions by pathologists, and all subjects signed informed consent. And the HPV16 *E6* primer was used to screen samples from women with cervical lesions or women with cervical squamous. A total of 66 cases of HPV16 infection in non-cancerous lesions or in squamous cell carcinoma were obtained. The samples were stored at -80°C for further processing. This study was conducted according to the guidelines of the Declaration of Helsinki.

### DNA extraction and HPV16 identification

DNA was extracted using an DP304 Genomic DNA extraction kit (Beijing Tiangen biochemical technology Company, Beijing, China) according to the kit instructions. The HPV16 *E6* primers were designed and polymerase chain reaction (PCR) was used to detect the HPV16 virus. The primer sequences for identification of HPV16 E6 were as follows: HPV16 E6-F, 5'-gaccagaaagttaccacag-3' and HPV16 E6-R, 5'-cacaacggtttgttattg-3' (F, forward primer; R, reverse primer)[16].

# PCR amplification and sequencing

The HPV16 *E6* and *E7* gene fragments were amplified by PCR. A 50µl reaction mixture containing 20 pmoles of each primer, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 100 mM Tris-HCl, pH 8.3, 0.1% Triton X-100, 50µM of each dNTP and, 1.8 U of HotStarTaq polymerase (Qiagen Inc.) and 5µl template DNA. The cycling program was 94°C for 5 min; 30 cycles of 55°C for 45 s, 72°C for 60 s, 94°C for 15 s; 55°C for 45 s, 72°C for 5 min. The sequencing primers are shown in Table 1 [16].

## Cell culture and transfection

The *E7* (prototype and mutant) and *E6 + E7* (prototype and mutant) gene was cloned into GV144 recombinant vector, respectively and verified by sequencing. In addition, GV144(-) vectors were used as controls. C33A cells were seeded at  $1 \times 10^6$  per well in a six-well plates 24 h before transfection. Subsequently, 2 µg *E7* and *E6 + E7* recombinant vector and the control vector were mixed with 5 µL of FuGENE HD (Roche), respectively. The FuGENE/DNA complexes were added to C33A cells. Transfected C33A cells were then incubated at 37°C in 5% CO<sub>2</sub> for 24 h.

## Western blotting

Cell lysates were prepared in RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40). Equal amounts of protein were fractionated by SDS-PAGE and blotted onto membranes. The membranes were incubated with primary antibodies against HPV16 E7 (bs-4623R, Bioss, 1:500), Caspase 3 Mouse Monoclonal antibody(#66470-2-Ig, Proteintech Group,1:1000), and antibodies against β-actin (66009-1-Ig, Abmart, 1:1,000), GAPDH (19F00412, Zhongshan Jinqiao Biotechnology, 1:1,000) were used as the loading control. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Bio-Rad, Hercules, CA, USA) were then applied, and signals were detected using Western ECL Substrate (Bio-Rad).

## Analysis of cell proliferation

Proliferation of C33A cells transfected by the *E7* (prototype and mutant), *E6 + E7* (prototype and mutant) GV144 construct and GV144 was determined using CCK-8 assays. C33A cells were seeded in 96-well plates at 3000 cells per well in DMEM(Gibco, USA)containing 10% FBS(Biological Industries, Israel). The cells were incubated for 24 h at 37°C. Then, 10 µL of CCK-8 solution(Dojindo, Japan) was added to each well and incubated for 4 h.

For analysis of clonal formation, C33A stably transfected cells, selected by G418, were inoculated into six-well plates, 1,000 cells per well, and after 14 d, colonies were fixed in 2 mL of 4% paraformaldehyde, followed by the addition of 0.4% crystalline purple staining dye. The number of clones with more than 50 cells were counted under a microscope.

## Scratch wound healing assay

Cells were seeded onto six-well plates ( $1 \times 10^6$  cells per well). When cells reached 90% confluence, a scratch was made across the cell monolayer. The cells were washed with PBS for three times to remove

detached cells and debris, and cultured in fresh medium without serums in an incubator of 5%CO<sub>2</sub> at 37°C. Then, size of wounds were observed and measured at the indicated times and photographed using an inverted tissue culture microscope at 40× magnification. Assays were performed at least three times, and data are presented as means ± SD.

## **Analysis of Cell apoptosis**

Apoptosis analysis was assessed with Annexin V-APC/7-AAD Apoptosis Detection kit (Joint Biology, Hangzhou). Cells were plated in a 6-well plate at a density of  $1 \times 10^6$  per well. After incubation for 48 h post-transfection, cells were harvested. Apoptosis was induced in accordance with the experimental protocol, the cells were washed with precooling PBS for three times to remove detached cells and debris, and incubated with 5 µl Annexin V-APC and 10 µl 7-AAD Staining Solution.

5000 cells/ml were taken to prepare a cell drop tablet for Immunohistochemistry (IHC). The primary antibody was replaced with phosphate-buffered saline (PBS) as a negative control. The primary antibody (Caspase 3 Mouse Monoclonal antibody, #66470-2-Ig, Proteintech Group) was diluted to 1:400.

## **Model of xenotransplanted tumors in nude mice**

For xenograft experiments,  $7 \times 10^6$  C33A cells stably expressing NV-GV144, HPV16 E7 or E6 + E7 prototype, E7 mutation or E6 + E7 co-mutations from recombinant expression vectors were injected subcutaneously into 5-week-old female BALB/c Nude mice (Beijing Vital River Laboratory Animal Technology Co., Ltd.). The tumor volumes were measured using digital calipers every 3 days and calculated using the equation: length (mm) × width<sup>2</sup> (mm) × 0.52. All animals were treated in accordance with institutional guidelines, and the experimental protocol was approved by the Ethics Committee guidelines of the First Affiliated Hospital, Shihezi University School of Medicine (approval number A2019-038-01).

## **GenBank accession number**

The HPV16 prototype (European prototype, GenBank accession: NC\_001526.2)

## **Statistical analysis**

All data were analyzed using SPSS 22.0 software (IBM, Armonk, NY, USA). The measurement data is represented by means ± SD. The data were normally distributed, displayed homogeneous variance and was subsequently analyzed by ANOVA. A *P*-value < 0.05 was considered statistically significant.

## **Results**

### **Mutation of the HPV16 E7 gene at nt 647 in cervical cancer and non-cancerous tissues**

In this study, a total of 66 cervical cancer and non-cancerous tissue samples were collected from women in Xinjiang with HPV16 infections. Among them, the A647G mutation was found in 4 out of 28 cases

(14.3%) with non-cancerous tissues and in 14 out of 38 cases (36.8%) with cervical cancer (Table 2).

## **Construction of HPV16 E7 prototype and HPV16 E7 mutant recombinant vectors**

According to the sequencing results, we found 18 cases with the A647G mutation in the E7 gene and 17 cases with the T178G mutation in the E6 gene (Table 2). Therefore, the following four recombinant vectors were designed and constructed (Fig. 1): HPV16 E7 prototype (647A), HPV16 E7-647G mutation, HPV16 E6/E7 prototype (E6-178T/E7-647A) and HPV16 E6-178G/E7-647G mutation. Sequencing of the recombinant vectors showed successful construction.

## **Western blot analysis of HPV16 E7 protein expression**

Transfection of NC-GV144, HPV16 E7 prototype (E7-647A)-GV144, HPV16 E7 mutant (E7-647G)-GV144, HPV16 E6/E7 prototype (E6-178T/E7-647A)-GV144 and HPV16 E6/E7 co-mutant (E6-178G/E7-647G)-GV144 was performed in C33A cells. As shown in the Fig. 2, HPV16 E7 protein expression bands were observed in the four transfected recombinant vector groups. Since C33A cells do not contain the HPV virus, there was no E7 protein expression, and no E7 bands were observed in the NC negative control. The results indicated that the recombinant vector was successfully transfected and could be used for subsequent experiments.

The HPV16 E7 prototype (E7-647A)-GV144 vector, HPV16 E7 mutant (E7-647G)-GV144 vector, HPV16 E6/E7 prototype (E6-178T/E7-647A)-GV144 vector and HPV16 E6/E7 mutant (E6-178G/E7-647G)-GV144 vector were transfected into C33A cells in 96-well plates. Non-transfected C33A cells were used as a blank control group, and the NC-GV144 vector group was used as a negative control group. OD values in the above groups at 450 nm were detected at 0 h, 24 h, 48 h, and 72 h after the addition of CCK-8 to determine the effect of transfection on proliferation of C33A cells. As shown in the Fig. 3A, compared with the NC-GV144 control group, E7 and E6/E7 prototypes significantly promoted the proliferation of C33A cells 72 h after transfection ( $P < 0.05$ ). The HPV16 E7-647G mutant group and the E6-178G/E7-647G co-mutant group showed significant cell proliferation 48 h and 72 h after transfection ( $P < 0.05$ ). The four recombinant expression vectors were then combined and compared (Fig. 3B). At 24 h and 48 h, the HPV16 E6-178G/E7-647G co-mutant group showed significantly increased proliferation of C33A cells ( $P < 0.05$ ). The results suggested that compared to NC-GV144 control group, the recombinant vector in the experimental group had a significant effect on the proliferation of C33A cells and that cell proliferation in the HPV16 mutant group was higher than in the HPV16 prototype group. In the mutant group, co-mutation of HPV16 E6-178G/E7-647G had the greatest impact on the proliferation of C33A cells.

## **Effects of HPV16 E7 mutation and E6/E7 co-mutation on clonal formation of C33A cells**

C33A cells stably transfected with NC-GV144, HPV16 E7 prototype (E7-647A)-GV144 vector, HPV16 E6/E7 prototype (E6-178T/E7-647A)-GV144 vector and HPV16 E6/E7 mutant (E6-178G/E7-647G)-GV144 vector were seeded on 6-well plates at a cell density of 1,500 cells per well for 2 weeks and then fixed with 4% paraformaldehyde. Colonies were stained with 0.1% Crystal Violet at room temperature and the number of cell colonies were counted at low power under a microscope. The results showed that compared with the control group of NC-GV144, the number of cell colonies in the four recombinant expression vector groups was significantly increased ( $P < 0.05$ ) and the number of cell colonies in the HPV16 mutant group was higher than in the HPV16 prototype group. Among the mutant groups, the co-mutation of HPV16 E6-178G/E7-647G had the greatest effect on clonal formation of C33A cells, indicating that the proliferation of C33A cells was greatest after co-mutation of HPV16 E6-178G/E7-647G (Fig. 3C and 3D).

## **Effect of HPV16 E7 mutation and E6/E7 co-mutation on migration of C33A cells**

The effects of HPV16 E7 prototype (E7-647A), HPV16 E7-647G mutation, HPV16 E6/E7 prototype (E6-178T/E7-647A) and HPV16 E6-178G/E7-647G co-mutation on C33A cell migration were studied by cell scratch experiment. C33A cells that were stably transfected with NC-GV144, HPV16 E7 prototype (E7-647A)-GV144, HPV16 E7 mutant (E7-647G)-GV144, HPV16 E6/E7 prototype (E6-178T/E7-647A)-GV144 and HPV16 E6/E7 mutant (E6-178G/E7-647G)-GV144 were inoculated in 6-well plates and scratched after reaching confluency. The gap healing width was observed at 0, 24 and 48 hours after the scratch, and the images were recorded. The results showed that 48 h after the scratch, the relative gap width of the HPV16 E7-647G mutant group and the HPV16 E6-178G/E7-647G mutant group was significantly smaller than that of the control group and the HPV16 prototype group. In the mutant groups, the relative gap width of HPV16 E6-178G/E7-647G co-mutation was smaller than that of the mutation group E7-647G, and the difference was statistically significant ( $P < 0.05$ ,  $n = 3$ ; Fig. 4A and 4B). These results suggested that HPV16 E7-647G and HPV16 E6-178G/E7-647G can promote cell migration ( $P < 0.05$ ) and that co-mutation of E6-178G/E7-647G promoted C33A cell migration to the greatest extent.

## **Effects of HPV16 E7 mutation and E6/E7 co-mutation on apoptosis of C33A cells**

We used flow cytometry to detect stable transfection of the GV144 empty vector, HPV16 E7 prototype (E7-647A) vector, HPV16 E7-647G mutant vector, HPV16 E6/E7 prototype (E6-178T/E7-647A) vector and HPV16 E6-178G/E7-647G co-mutation vector. The apoptosis rate of C33A cells in these five groups showed that the number of apoptotic cells in the NC-GV144 group was significantly higher than in the HPV16 E7 mutation group and the E6/E7 co-mutation group ( $P < 0.05$ ). Stable expression of the HPV16 prototype had no significant effect on apoptosis in C33A cells ( $P > 0.05$ ). This result suggested that both the HPV16 E7-647G mutation and E6-178G/E7-647G co-mutation could inhibit apoptosis of C33A cervical cancer cells (Fig. 4C and 4D). Immunohistochemistry and Western Blot assay were used to detect the expression of apoptosis protein Caspase 3 in each group. Western Blot analysis showed that the Caspase 3 expression of HPV16 E6 + E7 co-mutation group was decreased compared with the prototype and

HPV16 E7-647G-GV144 single point mutation group ( $P < 0.05$ ), as shown in Fig. 4E and 4F. Immunohistochemical of intracellular Caspase 3 in each group was consistent with the Western Blot results that the HPV16 E7 prototype group (Fig. 5C) was lower than the mutant group (Fig. 5D) and the HPV16 E6 + E7 prototype group (Fig. 5E) and the co-mutant group (Fig. 5F) were lower than the NC group (Fig. 5B) ( $P < 0.05$ ), the HPV16 E6 + E7 comutation group (Figure. 5F) showed decreased expression compared with the prototype group (Figure. 5E) and the HPV E7-647G-GV144 single point mutation group (Figure. 5D) ( $P < 0.05$ ). In conclusion, the HPV16 E7 mutation can inhibit the apoptosis of cervical cancer C33A cells, and the co-mutation of HPV16 E6-178G/ E7-647G-GV144 has a stronger effect(Figure. 5G).

## Xenograft tumor model in nude mice

C33A cells stably transfected with the four recombinant expression vectors were inoculated into nude mice. Tumor size was measured every 2–3 days. After 30 days, the nude mouse were killed through breaking neck (Fig. 6A-E), the tumors were removed and the average terminal tumor volume was determined with the following results: Control group, 52.8040.68 mm<sup>3</sup>; E7-647A prototype group, 59.9342.51 mm<sup>3</sup>; E7-647G mutation group, 70.9636.07 mm<sup>3</sup>; HPV16 E6-178T/E7-647A prototype group, 49.387.92 mm<sup>3</sup>; HPV16 E6-178G/E7-647G mutation group, 260.8210.27 mm<sup>3</sup>. Compared with the control group, there was no significant difference in tumor volume for E7-647A, E7-647G and E6-178T/E7-647A mice ( $P > 0.05$ ), while tumor size in the HPV16 E6-178G/E7-647G mutation group was significantly greater than the control group and other experimental groups ( $P < 0.05$ ; Fig. 6F-G). Mean weights of the terminal tumors were as follows: control group, 0.0740.031 g; E7-647A prototype group, 0.0920.015 g; E7-647G mutation group, 0.0910.122 g; E6-178T/E7-647A prototype group, 0.0900.029 g; E6-178G/E7-647G mutation group, 0.1380.043 g. Compared to the control group, the tumor weights in E7-647A, E7-647G and E6-178T/E7-647A mice were not significantly different ( $P > 0.05$ ), but the tumor weights in E6-178G/E7-647G mice were significantly greater than in the control group and other experimental groups ( $P < 0.05$ ). These results showed that the HPV16 E6-178G/E7-647G promoted the growth of cervical cancer to a greater extent than did the control and other experimental groups.

## Discussion

In the present study, we found that the rate of the *E7* gene A647G mutation in cervical cancer tissue was higher than in non-cervical cancer tissue(Table 2). In addition, HPV16 E7-A647G and E6-T178G co-mutations have been demonstrated in our previous studies [16], consistent with results of Ding et al.[17] The E7 oncoprotein encoded by HPV16 *E7* is 11 kDa in size and contains about 100 amino acids. E7 protein can promote the malignant transformation of cervical epithelial cells that are infected with HPV and maintain the malignant phenotype of cervical epithelial cells. The interaction between the *E7* gene and the retinoblastoma tumor suppressor Rb is one of the leading causes of cervical cancer. The region where the E7 protein binds to Rb is on amino acids 21–34, which include the 29th amino acid encoded by the nucleotide at position 647. The A647G mutation suppresses the physiological functions of Rb so as to maintain the HPV infection in the host over the long-term. Other studies have found that the incidence

of A645C (L28F) can reach 19% in the cervical cancer tissues of Korean women [18], and the mutation is also present in Japan and Italy [19, 20]. However, only one mutation at this site was found in our study, further suggesting that HPV mutations have regional characteristics.

In order to study the effect of the HPV16 E7 mutation on malignant progression of cervical cancer, we conducted cytological experiments to analyze and verify its function. Initially, four recombinant expression vectors of GV144, namely, HPV16 E7 prototype (E7-647A), HPV16 E7 mutant (E7-647G), HPV16 E6/E7 prototype (E6-178T/E7-647A) and HPV16 E6/E7 co-mutant (E6-178G/E7-647G) were designed and constructed according to sequencing results. HPV-negative C33A cervical cancer cells were selected for stable transfection, and the expression of HPV16 E7 protein in each group of cells after transfection was detected by western blot. The results suggested that the recombinant vector was successfully transfected (Fig. 2), and further *in vitro* cytology experiments were conducted to study and analyze the effect of different HPV16 E7 gene mutants on cervical cancer cells. The results of CCK-8, colony formation and proliferation experiments showed that among the four recombinant expression vectors, the co-mutation of HPV16 E6-178G/E7-647G had the strongest effect on proliferation of cervical cancer cells, followed by the mutation of HPV16 E7-647G. Flow cytometry detection showed that both HPV16 E6-178G/E7-647G co-mutation and the E7-647G mutation inhibited apoptosis of cervical cancer cells. Immunohistochemistry and Western Blot showed that the HPV16 E7 mutation can inhibit the apoptosis of cervical cancer C33A cells, and the co-mutation of HPV16 E6-178G/ E7-647G-GV144 has a stronger effect(Fig. 4E-F and Fig. 5). Through cell scratch experiments, we also observed that co-mutation of HPV16 E6-178G/E7-647G promoted the migration of cervical cancer cells to the greatest extent, followed by the mutation of E7-647G. These results thus demonstrated that the mutations changed the carcinogenic properties of the virus compared to the HPV16 prototype, and the mutations of these two HPV16 variants in this experiment promoted the malignant progression of cervical cancer cells, especially the co-mutation of HPV16 E6-178G/E7-647G. It has been reported that HPV16 E6 directly induces cervical cancer cell migration by regulating p53 signaling [21]. In order to further study the effect of the HPV16 E7-647G mutation and E6-178G/E7-647G co-mutation on cervical cancer cells, a xenograft tumor model was established in nude mice. Four days after inoculation of C33A cells stably transfected with the four recombinant expression vectors, tumor formation was observed in nude mice at the inoculation site. Tumor growth curve(Fig. 6H) analysis showed that 20 days after inoculation, the tumors of nude mice with the HPV16 E6-178G/E7-647G co-mutation showed significant differences from the other experimental groups. As could be seen from the appearance of the mice, the tumor volume was largest in the co-mutation group of HPV16 E6-178G/E7-647G. The tumors were removed and the terminal weight and volume of the tumors were analyzed, which showed that the HPV16 E6-178G/E7-647G polymorphic site promoted the growth of cervical cancer to a greater extent than did HPV16 E7-647G.

To summarize, our *in vivo* and *in vitro* experimental studies confirmed that the co-mutation of HPV16 E6-178G/E7-647G promoted the malignant progression of cervical cancer, and we intend to investigate the cancer-causing molecular mechanism of this mutation type.

# Conclusions

The genetic differences among HPV16 sub-types may be related to their carcinogenic potential. HPV mutations can differ in biology and etiology, leading to differences in tumor development and behavior. In our research, HPV16 E6-178G/E7-647G can thus promote the proliferation and inhibit the apoptosis of cervical cancer cells.

# Declarations

## Ethics approval and consent to participate

This study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving human subjects were approved by the First Affiliated Hospital, Shihezi University School of Medicine Ethics Review Board (approval number 2019-017-01), and informed consent was obtained from all of the participants.

All animals were treated in accordance with institutional guidelines, and the experimental protocol was approved by the Ethics Committee guidelines of the First Affiliated Hospital, Shihezi University School of Medicine (approval number A2019-038-01).

## Consent for publication

Not applicable.

## Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available because (The data belong to our group) but are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

Zm P designed the study. XZ and CZ performed the cell and animal studies, participated in the gene analysis and wrote the manuscript. Zz P and HX carried out specimen collection and DNA extraction. FJ and Hc L carried out HPV16 identification. WZ and Ht L performed the statistical analysis. DL and RS participated in the experimental design and revised the manuscript. All authors read and approved the final manuscript.

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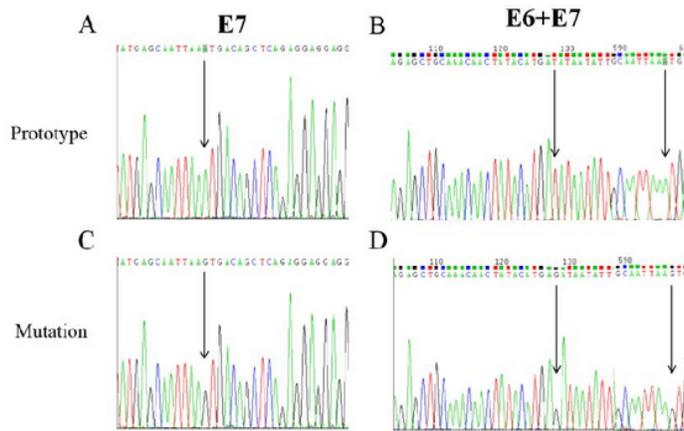
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## Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

## Figures

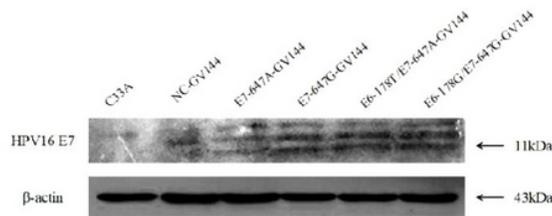
**Fig. 1.**



**Figure 1**

Sequencing maps of the GV144 recombinant expression vectors. A, HPV16 E7-647A. B, HPV16 E6-178T/E7-647A. C, HPV16 E7-647G. D, HPV16 E6-178G/E7-647G

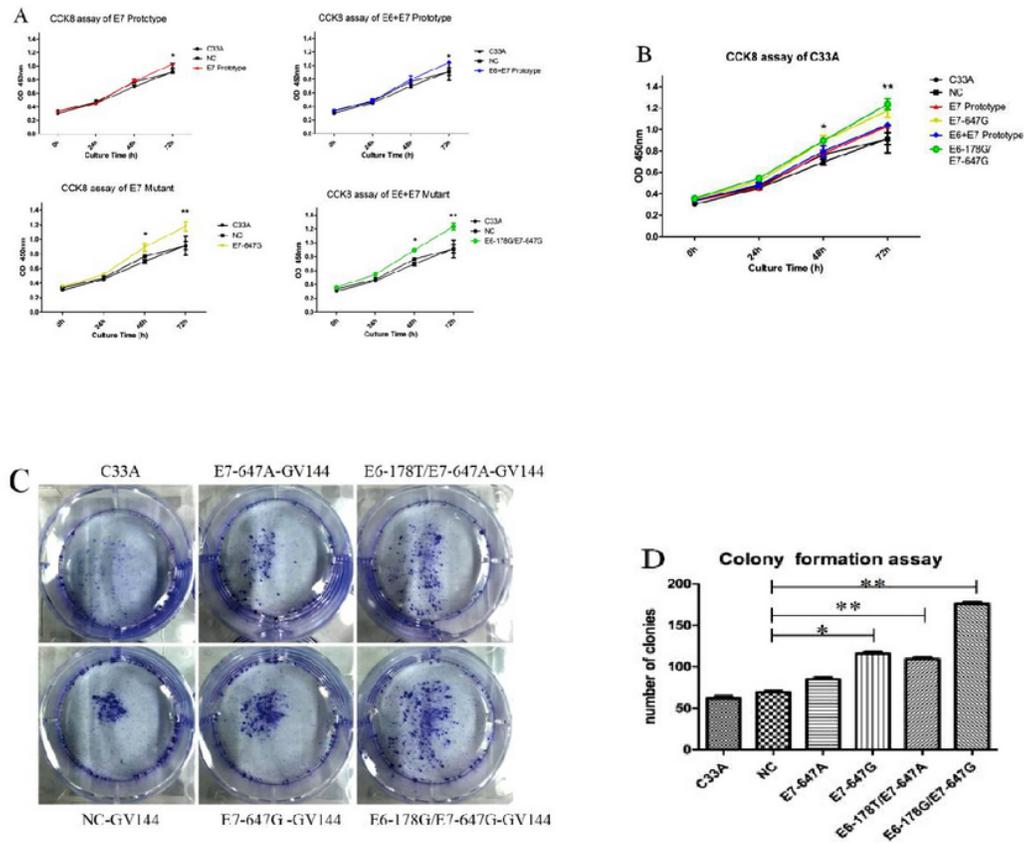
**Fig. 2.**



**Figure 2**

Protein expression of HPV16 E7. The proteins expression of HPV16 E7 in human cervical cancer cell lines C33A were detected by Western blotting after

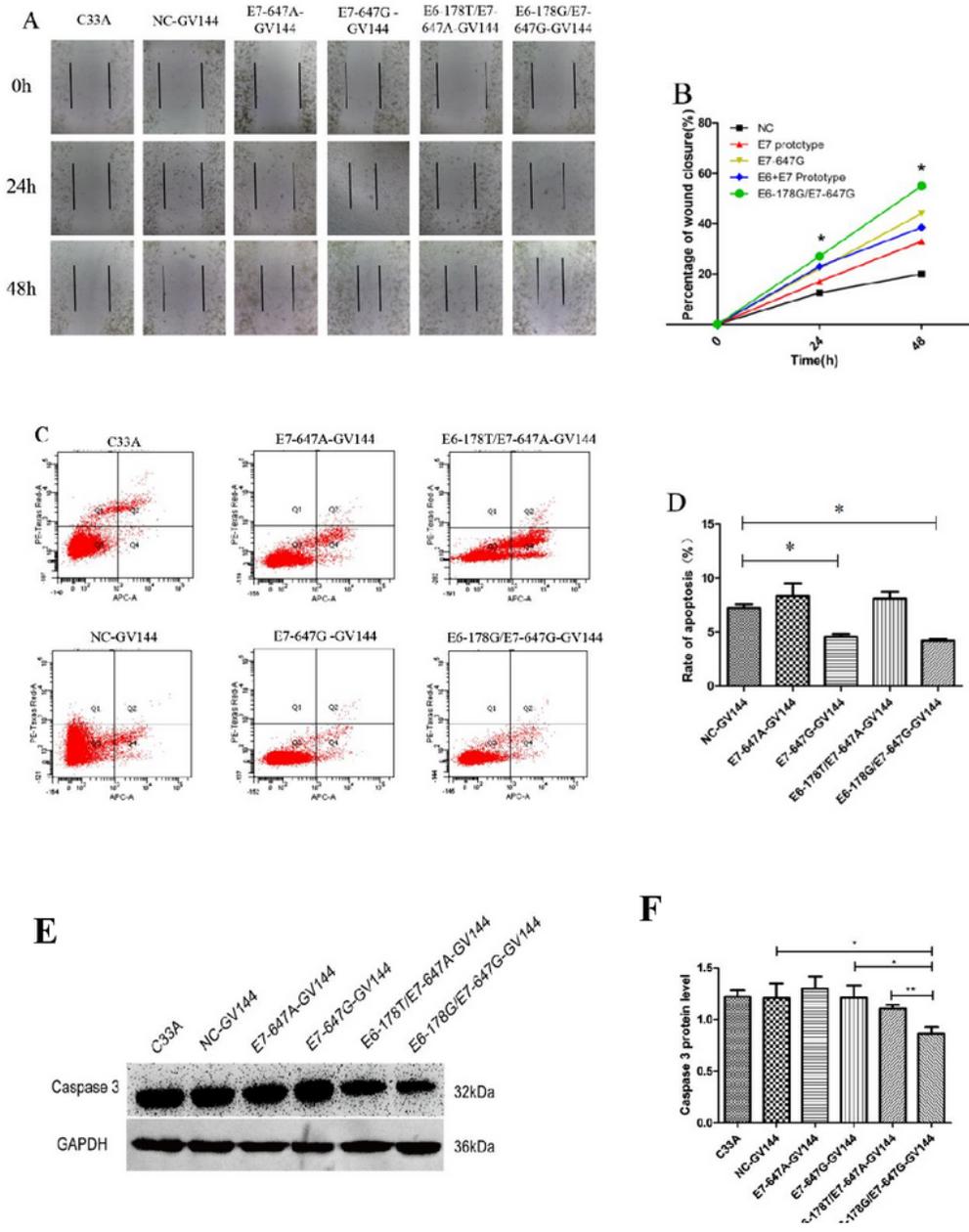
**Fig. 3.**



**Figure 3**

Effect of stable transfection of recombinant vectors on C33A cell proliferation. (A, B) CCK8 assays were used to evaluate the proliferation of C33A cells after transfection with NC-GV144, HPV16 E7 prototype (E7-647A)-GV144 vector, HPV16 E7 mutant (E7-647G)-GV144 vector, HPV16 E6/E7 prototype (E6-178T/E7-647A)-GV144 vector, HPV16 E6/E7 mutant (E6-178G/E7-647G)-GV144 vector. (C) Clonal formation of C33A cells after transfection with NC-GV144, HPV16 E7 prototype (E7-647A)-GV144 vector, HPV16 E7 mutant (E7-647G)-GV144 vector, HPV16 E6/E7 prototype (E6-178T/E7-647A)-GV144 vector, HPV16 E6/E7 mutant (E6-178G/E7-647G)-GV144 vector or after no transfection. (D) Statistical analysis of the number of clones formed. Data are representative of three independent experiments. \*P<0.05, \*\*P<0.01 vs

**Fig. 4.**



**Figure 4**

Effect of stable transfection of recombinant vectors on C33A cell migration and apoptosis. (A) Wound healing assay to analyze the effect of the HPV16 E7 mutation and E6/E7 co-mutation on C33A cell migration. (B) Statistical analysis of the percentage of wound closure. Data are representative of three independent experiments. \*P<0.05, \*\*P<0.01 vs control. (C) The effect of the HPV16 E7 mutation and E6/E7 co-mutation on apoptosis. (D) Statistical analysis of the rate of apoptosis. Data are representative

of three independent experiments. \* $P < 0.05$ . (E) Expression of apoptotic protein Caspase 3 in cells after transfection with recombinant GV144 vector. (F) Statistical analysis of the expression of Caspase 3 in each group of cells.

Fig. 5.

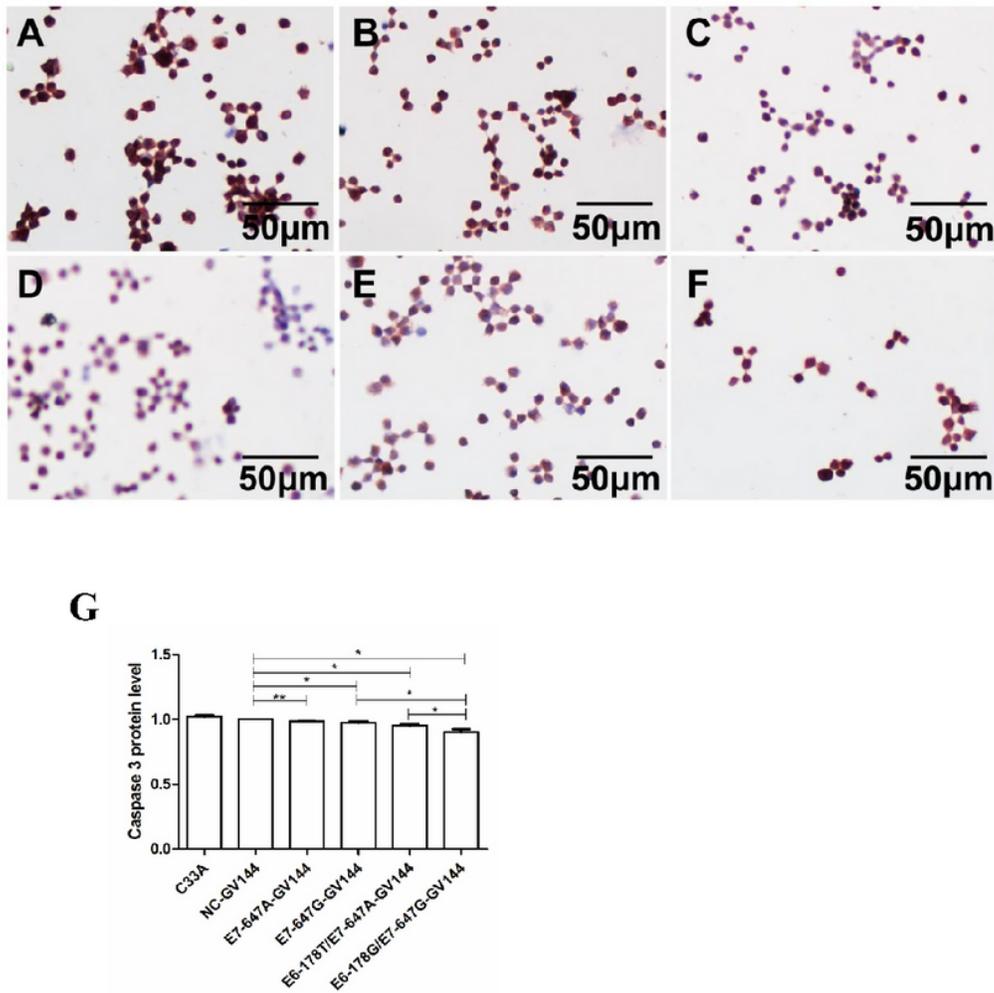
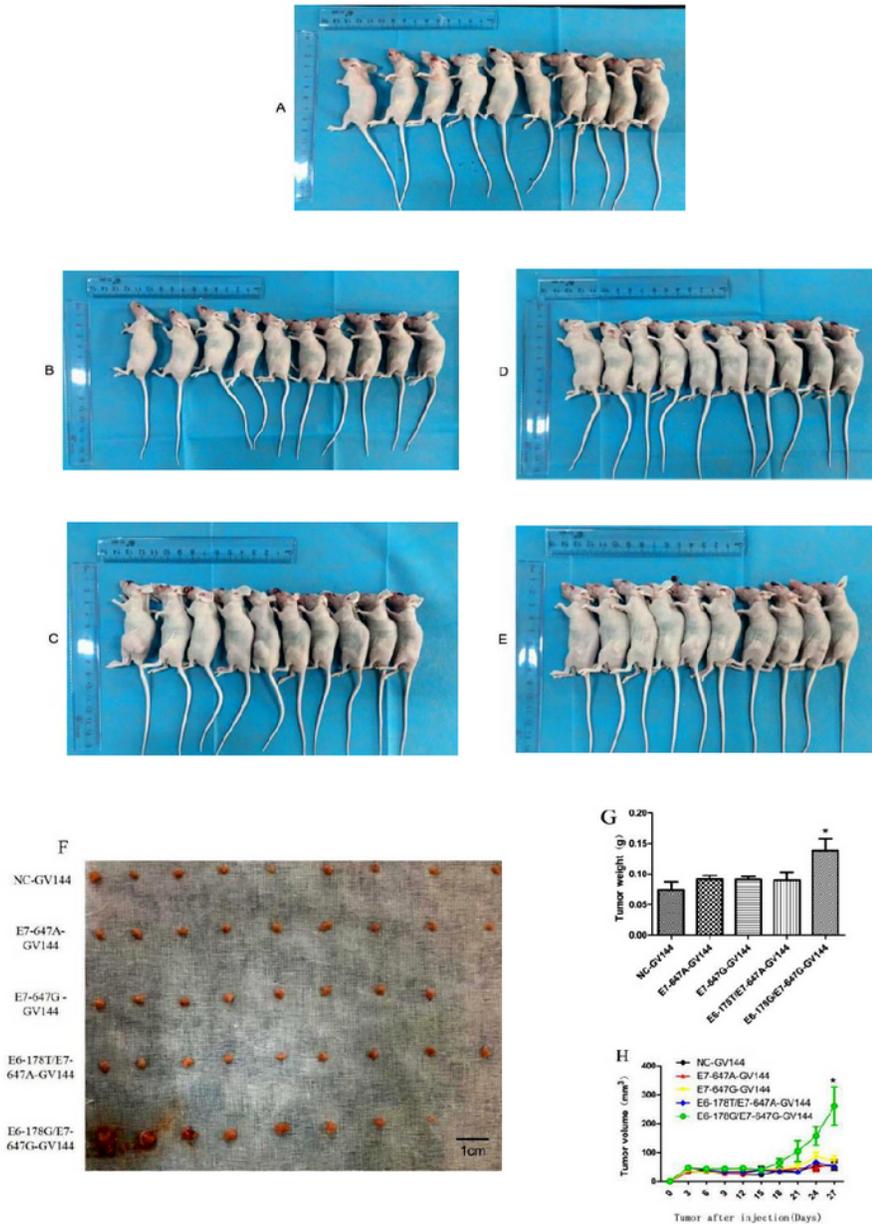


Figure 5

Expression of apoptotic protein Caspase 3 in cells after transfection with recombinant GV144 vector(100 × ). A, C33A. B, NC-GV144. C, E7-647A-GV144. D, E7-647G-GV144. E, E6-178T/E7-647A-GV144. F, E6-178G/E7-647G-GV144. G. Statistical analysis of the expression of Caspase 3 in each group of cells.

**Fig.6.**



**Figure 6**

HPV16 E6-178G/E7-647G promoted tumor growth in a nude mouse model. (A) Tumors in nude mice subcutaneously inoculated with NC-GV144. (B) Tumors in nude mice subcutaneously inoculated with E7-647A-GV144. (C) Tumors in nude mice subcutaneously inoculated with E7-647G-GV144. (D) Tumors in nude mice subcutaneously inoculated with E6-178T/647A-GV144. (E) Tumors in nude mice subcutaneously inoculated with E6-178G/647G-GV144. (F) Tumor size of five groups after tumorigenesis

in female athymic nude mice. (G) Quantitation of tumor weights after tumorigenesis in female athymic nude mice. (H) Quantitation of tumor volumes during tumorigenesis in female athymic nude mice.

## Supplementary Files

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- [3.Table.pdf](#)