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Original Article

Flt3L and GM-CSF enhance anti-tumor effect of HPV16/18 vaccine via increasing immune response

Yu Liu[1*], Haiyan Zhu[4*], Laiming Mo[2], Rui Xu[1], Xiangyun Li[1], Tian Li[3], Liang Zhao[5], Yi Ren[6], Rongying Ou[4], Yunsheng Xu[1]

Departments of [1]Dermatovenereology, [2]Clinical Laboratory, [3]Gynaecology and Obstetrics, The Seventh Affiliated Hospital, Sun Yat-Sen University, Shenzhen 518107, Guangdong, China; [4]Department of Gynaecology and Obstetrics, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, China; [5]Laboratory for Advanced Interdisciplinary Research, Institutes of Translational Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, China; [6]Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, FL 32306, USA. [*]Equal contributors.

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Abstract: Objectives: Cervical cancer is the second leading cause of cancer death in women, which is closely related to persistent infection with high-risk Human papillomavirus (HPV). Therefore, it is important to develop new adjuvants for HPV vaccines. This research aimed to establish two new adjuvants which can enhance the immune effect of vaccines. Materials and Methods: C57BL/6 mice were divided into 5 groups and immunized by intramuscular injection of plasmid once every 2 weeks, three times in all. The growth and metastasis of tumors in mice was observed by *in vivo* imaging system (IVIS). Then, the mice were sacrificed and the pathological changes of organs were observed. In addition, the lymphocyte suspension was used for CLT killing test. IFN-γ level and the number of splenocytes which secrete IFN-γ were detected. Additionally, the specific antibody level of HPV16/18 E6 E7 L1 L2 was also detected. Results: The constructed nucleic acid vaccines had no significant effect on both the physiological and biochemical indexes, while it significantly increased the survival period and survival rate of mice. Flt3L and GM-CSF enhanced the immune effect of HPV16/18 vaccine via increasing the specific antibodies and IFN-γ cytokines. Conclusions: These data suggested that Flt3L and GM-CSF enhanced the anti-tumor effect of vaccines via increasing immune response. Thereby, our findings may hope to provide new perspective for the treatment of cervical cancer.

Keywords: Cervical cancer, HPV16/18 vaccine, Flt3L, GM-CSF

Introduction

Cervical cancer (CC) is one of the major disease threats to women's health around the world. It is one of the most common malignant tumors which ranks second in the feminine unique tumors and the onset age increasingly reduced [1]. According to the Chinese Cancer Statistics Reports 2015, an estimated of 30, 500 deaths from CC occurred in China in 2015 [1]. CC is associated with the infection of Human papillomavirus (HPV) [1]. More than 99% CC para-carcinoma tissue contain HPV DNA, most of which is high-risk type of HPV16 and HPV18 [2]. Therefore, development of HPV16 and HPV18 vaccine is necessary for the treatment of CC.

The early protein E6 and E7 of HPV are the target antigens of most therapeutic vaccine [3]. The appropriate antigen and effective carrier are crucial in the immunotherapy. PVAX1 is a non-viral vector, which is the only carrier plasmid recommended by the U. S. Food and Drug Administration (FDA) that can be used in human experiments [4].

Nucleic acid vaccine can induce humoral and cellular immunity in immune response, while the efficiency of nucleic acid vaccine is relatively low [3]. Cytokine is an immunomodulatory substance produced by the body during the process of immune response, which has significant adjuvants immune effect [5]. In recent years, cytokine fms-like tyrosinekinase 3 ligan

(Flt3L) has been widely used as a nucleic acid vaccine adjuvant [6-8]. In addition, Flt3L is a good derived dendritic (DC) amplification agent which can interact with a variety of cytokines [9, 10]. Numerous studies have reported that Flt3L could enhance the cell-dependence and proliferation of T cell induced DC maturation in specific subpopulations as well [11, 12]. In addition, granulocyte monocyte colony stimulating factor (GM-CSF) is an important growth factor of hematopoietic cells which is produced by T cells [13]. Additionally, GM-CSF is involved in colony formation of monocyte, neutrophil, macrophage and eosinophil [13]. Moreover, GM-CSF enhances the immune response by increasing the number and function of antigen-presenting cells [13]. Besides, researchers have found that GM-CSF significantly increases the level of IgG as well as the expression of IFN-γ and IL-6 [14, 15].

B7 is a costimulatory molecule which certainly enhances the immune effect of nucleic acid vaccine [16]. T cells obtain stimulate signal via the interaction between CD28/CTLA4 and B7 on B cells and CD28 is a member of the immunoglobulin superfamily [17]. Linsley et al. proved that CD28 signaling pathway could be activated by B7 which results in promoting the secretion and proliferation of T-cells and costimulating CD28-B7 [18].

In this study, the combined vaccine expressing both the fusion antigen of upstream E6, E7 and adjuvant (Flt3L or GM-CSF) were investigate. In addition, the effects of these two immune-enhancing vaccines on tumorigenesis of cervical cancer were investigated.

Methods

Cell culture and experimental animals

The B16 cells were provided by Shanghai Fu Xiang Biotechnology (Shanghai, China). Cell stocks were maintained in 5% CO_2 at 37°C with RPMI 1640 medium containing 10% FBS. C57BL/6 mice were purchased from Laboratory Animal Center of Huazhong Agricultural University (Wuhan, China). The mice were fed under standard laboratory conditions and temperature-controlled room with a 12 h/12 h light-dark cycle. Standard chow and water were given in the laboratory.

Vector construction

According to the gene reference sequence and vector sequence in NCBI, the plasmids of PVAX1-IRES-GM-CSF-B7.1, PVAX1-IRES-Flt3-L, PVAX1-IRES-GM-CSF-B7.1-HPV16 E6 E7 L1 L2-Flt3-L, PVAX1-IRX1-IRES-HPES-HPV18 E6 E7 L1 L2-Flt3-L and PIRES-neo3-HPV18 E6 E7 L1 L2 were digested and sequenced.

Quantitative real-time PCR

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR (qRT-PCR) reaction was performed using the Taq-Man PCR Master Mix according to the manufacturer's protocol. $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression and β-actin was used as internal control. The incubation was initiated at 37°C for 15 min, followed by 95°C for 30 s, 60°C for 34 s for 40 cycles. The primers were listed in **Table 1**.

Western blot

The protein concentrations were detected using BCA protein assay kit (Beyotime, Shanghai, China). Then, equal amounts of protein extracts (30 μg) were loaded in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% SDS polyacrylamide gel. After that, the proteins were transferred to polyvinylidene fluoride membranes (PVDF, Thermo Fisher Scientific, Waltham, MA, USA) for 2 h and blocked with 4% skim milk powder in TBST for another 1 h. The membranes were incubated with primary antibodies overnight at 4°C: GM-CSF antibody (1:1000; Affinity, Cambridge, UK), B7.1 antibody (1:1000; Affinity), HPV16 E6/18 E6 antibody (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HPV16 E7 antibody (1:300; Santa Cruz Biotechnology), Flt3-L antibody (1:1000; Abcam, Cambridge, MA, USA), HPV16 L1 antibody (1:1000; Santa Cruz Biotechnology), HPV16 L2 antibody (1:1000; Santa Cruz Biotechnology), HPV18 E7 (1:1000; Abcam), HPV18 L1 (1:1000; Abcam), Luciferase antibody (1:1000; Abcam), β-actin antibody (1:1000; Wuhan doctorate Bioengineering, Wuhan, China). After washing with PBS, the PVDF membrane was incubated with anti-β-actin secondary antibody (1:5000; Abcam) at room temperature for 1 h. Finally, the immuno-

Table 1. The sequence of primers

Gene	Forward Primer	Reverse Primer
GM-CSF	GGCAGCCTCACCAAGCTCAAG	GCAGTCAAAGGGGATGACAAG
B7.1	GCTGGCTGGTCTTTCTCAC	ACTCGTATGTGCCCTCGTC
HPV16 E6	CAGGAGCGACCCAGAAAGT	AACGGTTTGTTGTATTGCTGTTC
HPV16 E7	AGGAGGATGAAATAGATGG	TTGTACGCACAACCGAAGC
HPV16 L1	GCAGGAACATCCAGACTAC	CACGACCTACCTCAACACC
HPV16 L2	TTGTAACCACTCCCACTAA	AATGCTGGCCTATGTAAAG
HPV18 E6	CAGGAGCGACCCAGAAAGT	AACGGTTTGTTGTATTGCTGTTC
HPV18 E7	AGGAGGATGAAATAGATGG	TTGTACGCACAACCGAAGC
HPV18 L1	GCAGGAACATCCAGACTAC	CACGACCTACCTCAACACC
HPV18 L2	TTGTAACCACTCCCACTAA	AATGCTGGCCTATGTAAAG
Flt3-L	AGCTGTCTGACTACCTGCTTCA	GATGTTGGTCTGGACGAAGC
Luciferase	TCAAAGAGGCGAACTGTGTG	GGTGTTGGAGCAAGATGGAT
β-actin	CACGATGGAGGGGCCGGACTCATC	TAAAGACCTCTATGCCAACACAGT

reactivity was detected using the ECL reagent (Santa Cruz Biotechnology).

Immunization of mice

C57BL/6 mice (6-8 weeks) were randomly divided into 5 groups (5 mice/group). The nucleic acid vaccine or control plasmid PVAX1-IRES was prepared with sterilized saline at doses of 50 μg/mouse for nucleic acid vaccine and 50 μg/mouse for adjuvant, and the injection volume was 100 μl. The mice were immunized by intramuscularly injection of nucleic acid vaccine once every 2 weeks for 3 times. The blank group was injected with normal saline, the negative control (NC) group was injected with PVAX1-IRES control plasmid, the HPV16 group was injected with PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1, the HPV18 group was injected with PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L, and last group (HPV16+HPV18) was the combination group which was injected with PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 and PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L.

Evaluation of safety of vaccine in animals

After 10 days of last injection, the plasma of mice was obtained. The levels of RBC, HB, HCT, MCV, MCH, MCHC, PLT, WBC, RDW-CV, RDW-SD, ALT, AST, ALP, T-BIL, BUN, Cr, TP, ALB and GLU in plasma were measured by automatic biochemical analyzer (Rayto, Shenzhen, China). Then, the mice were sacrificed and the heart, brain, kidney, spleen, lung and liver were immediately taken out and fixed with 10% formaldehyde solution. The tissues were then dehydrated, paraffin-embedded and sliced (4 μm thick). HE staining was performed to observe the histopathological changes under optical microscope.

Stable transfection

The stable B16 cells transfected with PIRES-neo3, PIRES-neo3-HPV16 E6 E7 and PIRES-neo3-HPV18 E6 E7 were screening by exposure to G418 (0, 400, 500, 600, 700, 800 μg/ml). The efficiency of transfection was verified by qRT-PCR and Western blot.

In vivo imaging system (IVIS) investigation

After immunizations, the mice were subcutaneously inoculated with 2×10^6 (0.1 ml) cells of HPV16 E6 E7 L1 L2 (luciferase), HPV18 E6 E7 L1 L2 (luciferase). After 8 weeks of inoculation, the mice were anaesthetized by intraperitoneal injection of 3% sodium pentobarbital and observed by IVIS (Perkin Elmer, Waltham, China). The survival of mice was observed for 15 weeks. In the end of study, the heart, liver, spleen, lung, kidney and brain tissues of mice were collected for observing the metastasis of tumors.

Cytotoxic lymphocyte (CTL) killing test

The spleens of mice in each group were grinded with stainless steel wire mesh. The single cell suspension was slowly loaded into lymphocyte separating solution. After centrifugation (2000 *g)* for 10 min, the lymphocytes were collected. The lymphocytes were washed twice with RPMI 1640 and co-cultured with B16 (HPV16 E6 E7 L1 L2) or with B16 (HPV18 E6 E7 L1 L2) for 5 days at 10:1, 20:1 or 40:1 ratio. In addition, 20 U/ml recombinant mouse IL-2 (rmIL-2) and 10 μg/ml ConA were also added to the medium. B16 cells stably transfected with HPV16 E6 E7 L1 L2 or HPV18 E6 E7 L1 L2 were pretreated with mitomycin C at a concentration of 50 μg/ml for 1 h, and then co-incubated with spleen cells. The killing activity of CTL to target cells

was calculated according to the following formula.

Percent cytotoxicity = Experimental value - Effector spontaneous value - Target spontaneous value × 100% Target maximum value - Target spontaneous value.

Detection of antigen-specific immune responses

Mice serum was collected at 4th, 8th, 12th, 16th and 20th weeks after the first immunization by Tail-cutting method for determination of specific antibody. The recombinant HPV16 E6 E7 Antibody (Ab) was coated in 96-well plate at 37°C for 2 h, sealed with 10% calf serum for 1 h, and then reacted with different dilutions of the tested serum for 1 h. Finally, added with excessive horseradish peroxidase labeled goat anti-mouse reactions for 1 h. After completing the above reactions, the color developer A and B were added to the dark box and observed every 10 min until the negative control (blank serum) was slightly better. Finally, 2 M sulphuric acid was added to stop the reaction and the OD value (492 nm) was measured.

Preparation of spleen lymph node cells and induction of IFN-γ cytokines

Three weeks after the last immunization, the mice were sacrificed and the spleen lymphocyte cells were collected. Then, the lymphocyte cells co-cultured with B16 (HPV16 E6 E7 L1 L2) or with B16 (HPV18 E6 E7 L1 L2) for 24 and 72 h, respectively. After that, the cell supernatant was collected and the level of IFN-γ was detected by ELISA Kit (Excellbio, Shanghai, China), according to the instructions of manufacturer.

Spleen lymphocyte cells detection

Three weeks after the last immunization, the mice were sacrificed and the spleen lymphocyte cells were collected. Then, the lymphocyte cells co-cultured with B16 (HPV16 E6 E7 L1 L2) or with B16 (HPV18 E6 E7 L1 L2) for 72 h, respectively. After that, the number of spleen lymphocyte cells was detected with ELISpot [19], according to the instructions of manufacturer.

Statistical analysis

Each group were performed at least three independent experiments and all values were expressed as the mean ± standard deviation (SD). The comparison between two groups was analyzed by Student's t-test. The comparisons among multiple groups were made with one-way analysis of variance (ANOVA) followed by Turkey's test (Graphpad Prism7). For all tests, values of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Construction of vectors

According to the sequences of gene and vectors in NCBI, the plasmids of PVAX1-IRES-GM-CSF-B.7.1, PVAX1-IRES-Flt3-L, PCI-HPV16 E6 E7 L1 L2, PCI-HPV18 E6 E7 L1 L2, PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1, PVAX1-IRES-HPV18 E6 E7 L1 L2-Flt3-L, PIRES-neo3-HPV16 E6 E7 L1 L2, PIRES-neo3-HPV18 E6 E7 L1 L2, PGEX-4t-3-HPV16 E6 E7 L1 L2 and PGEX-4t-3-HPV18 E6 E7 L1 L2 were designed and constructed. As indicated in **Figure 1** and **Table 2**, the product sizes of the recombinant plasmids PCI (**Figure 1A-C**), PVAX1-IRES (**Figure 1D-G**), PIRES-neo3 (**Figure 1H and 1I**) and PGEX-4t-3 (**Figure 1J-L**) were consistent with the expected product sizes. Additionally, the overexpression of plasmid was detected by qRT-PCR, Western blot and ELISA. The results indicated the gene expressions of GM-CSF and B7.1 were significantly increased in B16 cells after PVAX1-IRES-GM-CSF-B7.1 transfection (**Figure 2A**). Additionally, gene expression of Flt3L in B16 cells were increased significantly after transfection of PVAX1-IRES-Flt3-L, and the level of Flt3L in supernatant was also increased significantly (**Figure 2B** and **2C**). In addition, the expression of HPV16 E6 E7 L1 L2 in B16 cells was significantly increased after transfection of PIRES-neo3-HPV16 E6 E7 L1 L2 (**Figure 2D**). Similarly, PVAX1-IRES-GM-CSF-B7.1-HPV16 E6 E7 L1 L2 greatly increased the expression of HPV16 E6 E7 L1 L2 in B16 cells (**Figure 2E**). Besides, the expression of HPV18 E6 E7 L1 L2 in B16 cells was obviously upregulated by PIRES-neo3-HPV18 E6 E7 L1 L2 (**Figure 2F**). On the other hand, gene expression of HPV18 E6 E7 L1 L2 in B16 cells was notably enhanced in the presence of PVAX1-IRES-HPV18 E6 E7 L1 L2-Flt3-L (**Figure 2G**). Meanwhile, the results of q-PCR and ELISA were further confirmed by western blot (**Figure 3A-F**). All these data indicated that the fusion genes of PVAX1-IRES-GM-CSF-B7.1, PVAX1-

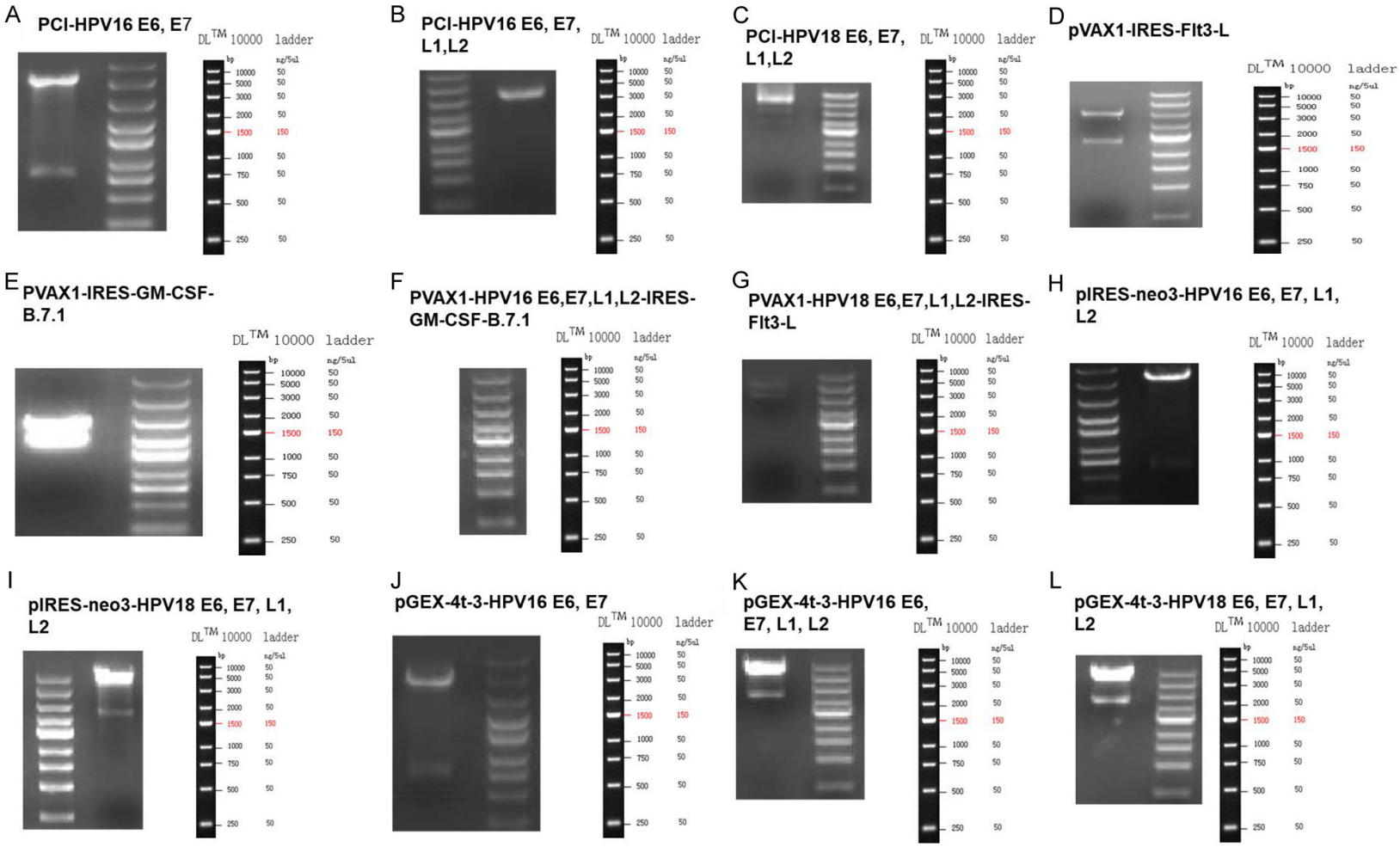

Figure 1. Plasmids with over expression efficiency were constructed successfully. Validation of PCI-HPV16 E6 E7 (A), PCI-HPV16 E6 E7 L1 L2 (B), PCI-HPV18 E6 E7 L1 L2 (C), PVAX1-IRES-Flt3-L (D), PVAX1-IRES-GM-CSF-B.7.1 (E), PVAX1-IRES-GM-CSF-B.7.1-HPV16 E6 E7 L1 L2 (F), PVAX1-IRES-HPV18 E6 E7 L1 L2-Flt3-L (G), PIRES-neo3-HPV16 E6 E7 L1 L2 (H), PIRES-neo3-HPV18 E6 E7 L1 L2 (I), pGEX-4t-3-HPV16 E6 E7 (J), PGEX-4t-3-HPV16 E6 E7 L1 L2 (K) and PGEX-4t-3-HPV18 E6 E7 L1 L2 (L) vaccine.

Table 2. Vectors information

Plasmid name	Restriction site	Gene length	Anti-resistance
PCI-HPV16 E6 E7	EcoR I/Xba I	789	Amp
PCI-HPV16 E6 E7 L1 L2	Xho I/Xba I	3735	Amp
PCI-HPV18 E6 E7 L1 L2	Xho I/Not I	3914	Amp
pVAX1-IRES-Flt3-L	BamH I/EcoR I	720	Amp
PVAX1-IRES-GM-CSF-B.7.1	BamH I/EcoR I	1892	Kan
PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1	Hind III/Xho I	5615	Kan
PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L	Hind III/Xho I	5214	Kan
pIRES-neo3-HPV16 E6 E7 L1 L2	Nhe I/Not I	3737	Amp
pIRES-neo3-HPV18 E6 E7 L1 L2	EcoR I/Not I	3914	Amp
pGEX-4t-3-HPV16 E6 E7	BamH I/EcoR I	789	Amp
pGEX-4t-3-HPV16 E6 E7 L1 L2	Sa II/Not I	3893	Amp
pGEX-4t-3-HPV18 E6 E7 L1 L2	Sa II/Not I	3914	Amp

IRES-Flt3-L, PVAX1-IRES-GM-CSF-B7.1-HPV16 E6 E7 L1 L2, PIRES-neo3-HPV16 E6 E7 L1 L2, PVAX1-IRES-HPV18 E6 E7 L1 L2-Flt3-L and PIRES-neo3-HPV18 E6 E7 L1 L2 could be expressed normally and the plasmids were constructed successfully.

Effects of nucleic acid vaccines on blood and biochemical indexes in immunized mice

In order to evaluate the safety of the vaccine, the blood physiology and biochemistry of immunized mice were tested. The results indicated that HPV16 or/and HPV18 had no significant effects on WBC (**Figure 4A**), RBC (**Figure 4B**), HGB (**Figure 4C**), MCV (**Figure 4D**), MCH (**Figure 4E**), MCHC (**Figure 4F**), PLT (**Figure 4G**), HCT (**Figure 4H**), RDW-CV (**Figure 4I**) or RDW-SD (**Figure 4J**) of mice. Similarly, the levels of ALT (**Figure 5A**), AST (**Figure 5B**), ALP (**Figure 5C**), T-BIL (**Figure 5D**), BUN (**Figure 5E**), CR (**Figure 5F**), TP (**Figure 5G**), ALB (**Figure 5H**) or GLU (**Figure 5I**) in mice were limitedly affected by HPV16 or/and HPV18. Taken together, HPV16 or/and HPV18 had no significant effect on blood physiology and biochemical indexes of mice. These results suggested that the constructed nucleic acid vaccines had no significant toxic side effect.

Effect of nucleic acid vaccines on pathological indexes of visceral tissue in mice

Next, in order to evaluate the safety of the vaccine, the histopathological indexes of immunized mice were tested. **Figure 6** showed that there was no significant difference in the morphology of brain, heart and kidney tissues compared to control. This data indicating that brain, heart and kidney tissues were not significantly damaged. However, there were a few inflammatory vacuoles in liver tissues. Slight thickening of alveolar wall, shrinkage of alveolar cavity, infiltration of inflammatory cells in lung tissues and shrinkage of lymphoid follicles in spleen tissues were observed. These results showed that these nucleic acid vaccines exhibited a high safety and had no genetic toxicity.

E6, E7, L1, L2 gene transfection

Next, in order to detect the efficacy of transfections, the levels of E6, E7, L1, L2 gene and proteins in B16 cells were measured with qRT-PCR and Western blot and the proliferation of B16 was detected by using MTT assay. E6 and E7 proteins are two of the important members in the malignant transformation of HPV16 and HPV18. The late proteins L1 and L2 play important role in the capsid structure of the virus. The results indicated that E6, E7, L1, L2 gene expressions in B16 cells were significantly increased after transfection of pIRES-neo3-HPV16 E6 E7 L1 L2 and PIRES-neo3-HPV18 E6 E7 L1 L2 (**Figure 7A-D**). Moreover, the levels of luciferase protein were significantly increased in PIRES-neo3-HPV16 E6 E7 L1 L2+pGL3-luc and PIRES-neo3-HPV18 E6 E7 L1 L2+pGL3-luc groups compared to PIRES-neo3 (**Figure 7E**). Besides, western blot data suggested that E6, E7, L1, L2 protein levels were notably increased in cells transfected with pIRES-neo3-HPV16 E6 E7 L1 L2 and PIRES-neo3-HPV18 E6

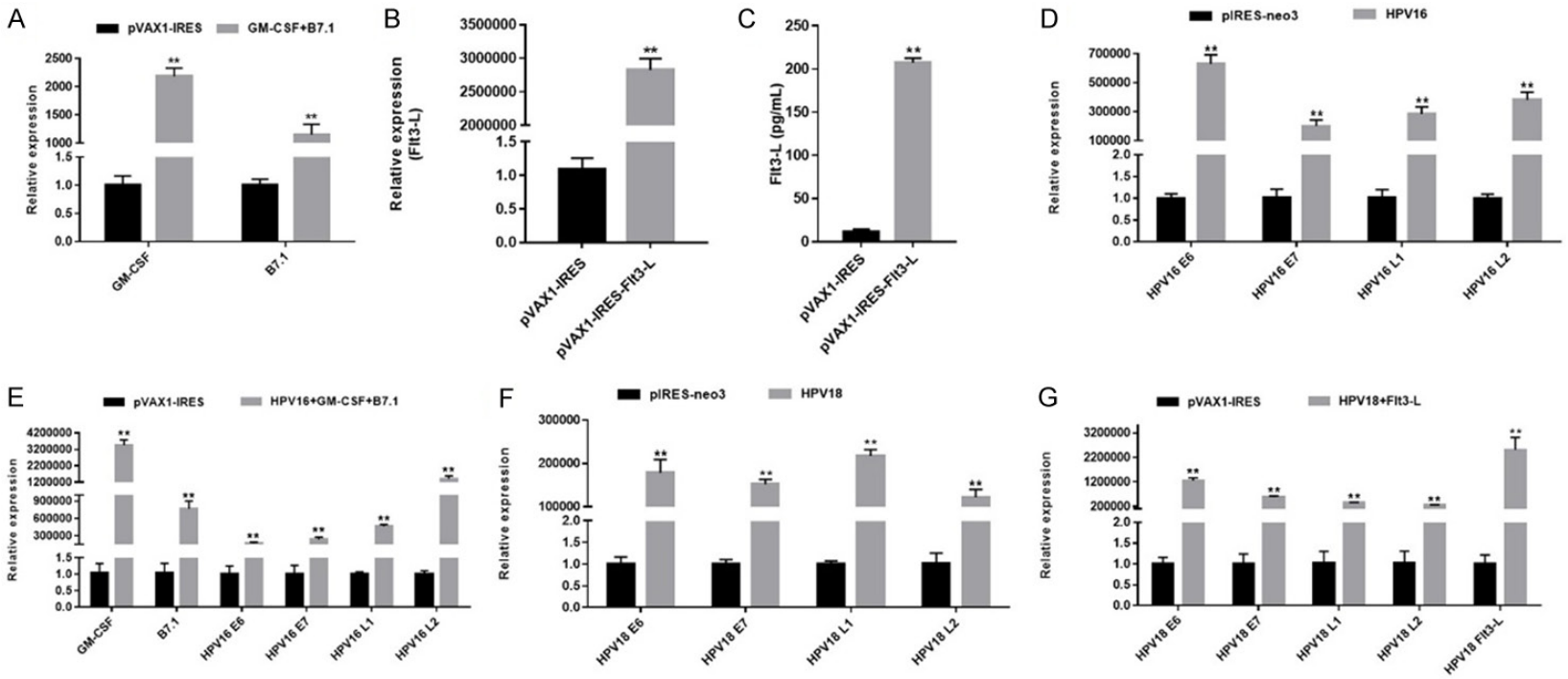

Figure 2. Target genes were detected by q-PCR. Gene expression of PVAX1-IRES-GM-CSF-B.7.1 (A), PVAX1-IRES-Flt3-L (B), PIRES-neo3-HPV16 E6 E7 L1 L2 (D), PVAX1-IRES-GM-CSF-B.7.1-HPV16 E6 E7 L1 L2 (E), PIRES-neo3-HPV18 E6 E7 L1 L2 (F), PVAX1-IRES-HPV18 E6 E7 L1 L2-Flt3-L (G) in B16 cells was detected by qRT-PCR. Level of Flt3-L (C) in B16 cells transfected by PVAX1-IRES-Flt3-L was detected by ELISA. $^{**}P < 0.01$ vs. PVAX1-IRES or pIRES-neo3 group.

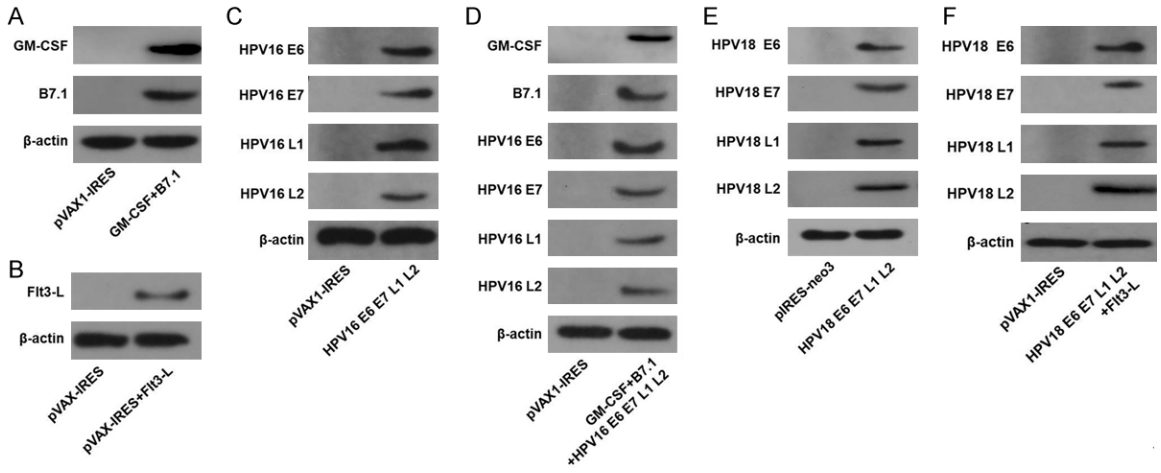

Figure 3. Target proteins were evaluated by Western blot. Protein expression of PVAX1-IRES-GM-CSF-B.7.1 (A), PVAX1-IRES-Flt3-L (B), PIRES-neo3-HPV16 E6 E7 L1 L2 (C), PVAX1-IRES-GM-CSF-B.7.1-HPV16 E6 E7 L1 L2 (D), PIRES-neo3-HPV18 E6 E7 L1 L2 (E), PVAX1-IRES-HPV18 E6 E7 L1 L2-Flt3-L (F) in B16 cells was detected by Western blot.

E7 L1 L2 compared pIRES-neo3 (**Figure 7F** and **7G**). Meanwhile, MTT assay demonstrated that 500 μg/ml G415 should be selected of use for stable cell strain selection (**Figure 7H**). Finally, the OD value of B16 cells was significantly increased in the presence of pGL3-luc (pGL3), PIRES-neo3-HPV16 E6 E7 L1 L2+pGL3-luc (HPV16+pGL3) or PIRES-neo3-HPV18 E6 E7 L1 L2+pGL3-luc (HPV18+pGL3), compared with PIRES-neo3 (NC) (**Figure 7I**). This result demonstrated that the stable strain was constructed. These results indicated that stable transfections of PIRES-neo3, PIRES-neo3-HPV16 E6 E7 L1 L2+pGL3-luc, PIRES-neo3-HPV18 E6 E7 L1 L2+pGL3-luc were successfully constructed.

The two purified proteins were obtained

The transformed vectors PGEX-4t-3-HPV16 E6 E7 L1 L2 and PGEX-4t-3-HPV18 E6 E7 L1 L2 were induced by IPTG, purified and detected to provide antigens for subsequent animal experiments. As shown in Supplementary Figure 1A and 1B, the molecular weight of protein was about 170 kD, which was in accordance with the theoretical size of HPV16 E6 E7 L1 L2 and HPV18 E6 E7 L1 L2 proteins. Combining with the previous sequencing results, it was concluded that these two proteins were successfully purified. The quantitative results of BCA showed that the HPV16 E6 E7 L1 L2 was 3.54 ± 0.12 mg/ml and the other one was 3.70 ± 0.05 mg/ml.

Vaccines exhibited anti-tumor effects in vivo

For the aim to investigate the anti-tumor effect of nucleic acid vaccine *in vivo*, tumor metastasis and survival rate of mice was detected. As indicated in **Figure 8A**, the survival rate of blank group and NC group was 0%, while that of HPV16, HPV18 and HPV16+HPV18 group was 20%, 20% and 30%, respectively. Moreover, the growth and metastasis of tumors in blank and PVAX1-IRES (NC) group were not significantly different from each other. The tumor growth of mice in PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 (HPV16) and PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L (HPV18) group was significantly slowed down, and the metastasis of tumors was notably inhibited by the combination of PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 and PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L (HPV16+HPV18) (**Figure 8B**). In addition, FLT3L and GM-CSF notably enhanced the anti-tumor effect of the HPV-16/18 E6/E7 vaccine on hepatic metastasis; however, there was no significant difference in the morphology of the heart, lung, spleen, kidney or brain between the groups (**Figure 8C**). In summary, all these results suggested that GM-CSF and Flt3L enhanced the anti-tumor effect of HPV16/HPV18 vaccine.

Vaccines induced significant immune response

Next, to evaluate the immune effect of the vaccine, the levels of specific antibodies and IFN-γ cytokine in mice were detected by ELISA and

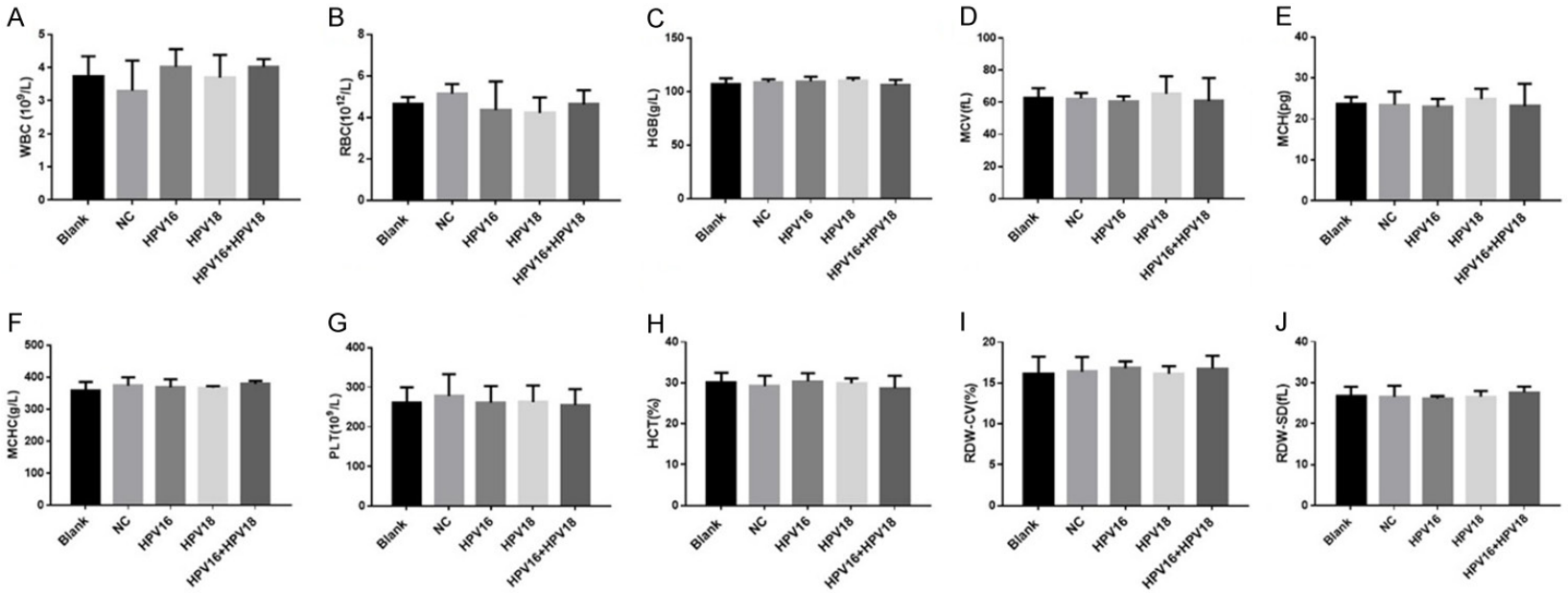

Figure 4. Nucleic acid vaccines had no effect on blood routine test in mice. Levels of WBC (A), RBC (B), HGB (C), MCV (D), MCH (E), MCHC (F), PLT (G), HCT (H), RDW-CV (I) and RDW-SD (J) in plasma of mice was detected automatic biochemical analyzer.

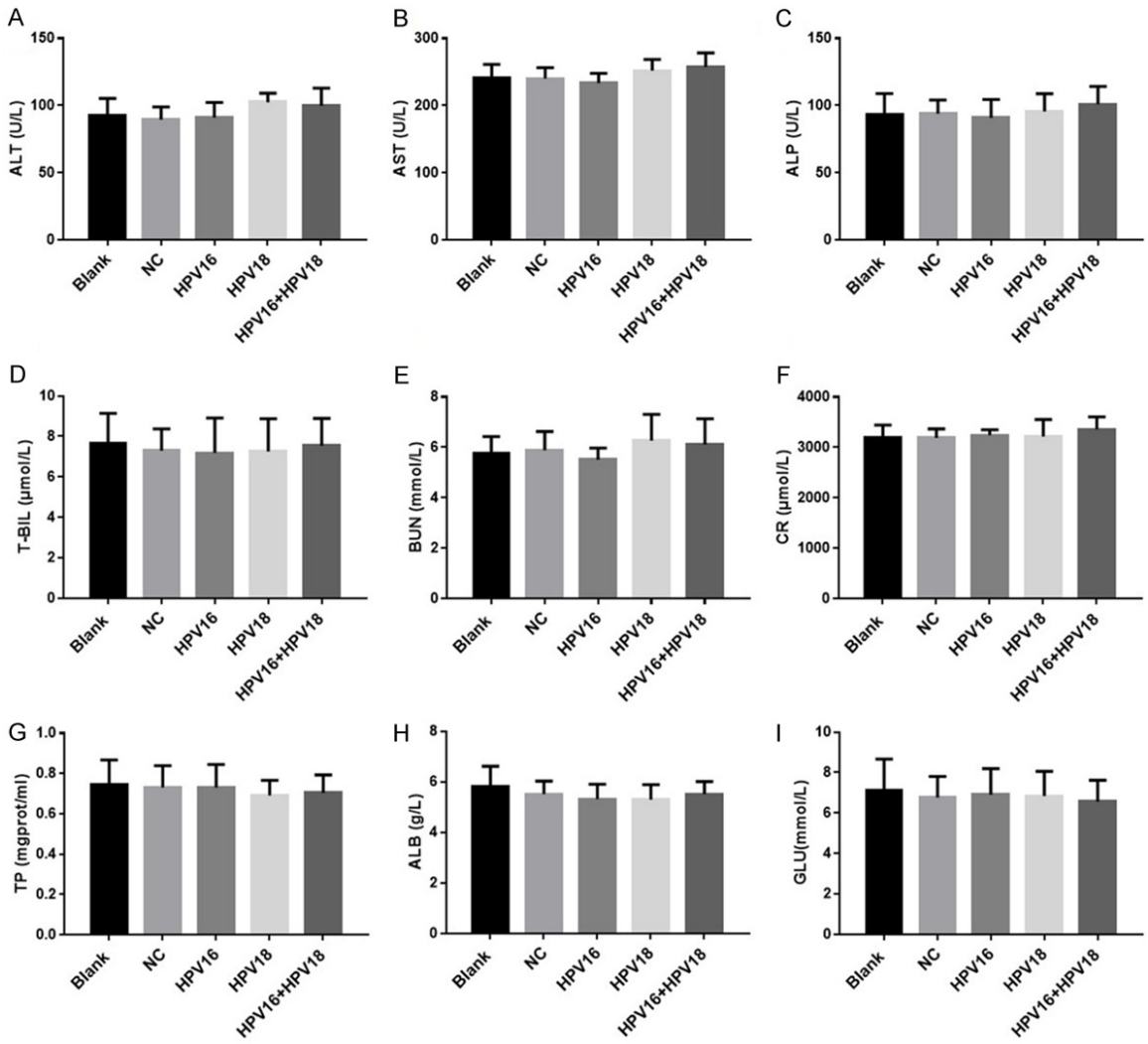

Figure 5. Nucleic acid vaccine does not affect blood biochemical index in mice. Levels of ALT (A), AST (B), ALP (C), T-BIL (D), BUN (E), CR (F), TP (G), ALB (H) and GLU (I) in plasma of mice was detected automatic biochemical analyzer.

ELISpot. As shown in **Figure 9A**, there was no significant difference in titer of serum specific antibodies between PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 (HPV16) and PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L (HPV18) group, while the titer of serum specific antibodies was significantly increased in these two groups compared to PVAX1-IRES (NC) group. In addition, the titer of serum specific antibodies was further elevated in PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 plus PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L (HPV16+HPV18) group. These results showed that GM-CSF and Flt3L could enhance the ability of nucleic acid vaccine for serum specific antibodies production. Moreover, when stimulated by HPV16/HPV18 E6 E7 L1 L2, the IFN-γ level in HPV16 or HPV18 group was significantly increased compared with NC group, which was further elevated by HPV16+HPV18 (**Figure 9B** and **9C**). In ELISpot detection, the number of spleen cells secreting IFN-γ in HPV16 or HPV18 group was increased compared to NC group, which was further increased by HPV16+HPV18 (**Figure 9D** and **9E**). When stimulated by HPV16 or HPV18 the number of IFN-γ in both HPV16 and HPV18 group were significantly elevated, which was further increased in HPV16+HPV18 group (**Figure 9D** and **9E**). Altogether, GM-CSF and Flt3L significantly enhanced immune response of HPV16 E6 E7 L1 L2 and HPV18 E6 E7 L1 L2 vaccines.

Vaccines exhibited a significant tumor killing effect

Finally, to confirm the killing effect of nucleic acid vaccine on tumors, the killing activity of

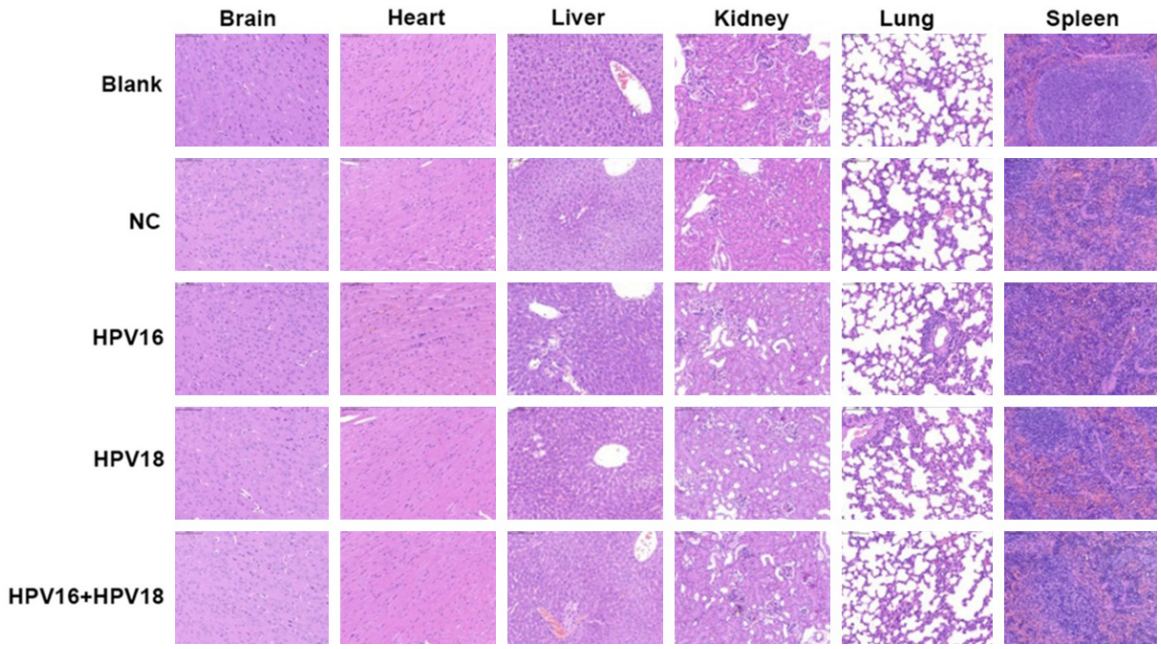

Figure 6. HE staining of visceral tissues in mice. Heart, brain, kidney, spleen, lung and liver were immediately taken out and fixed with 10% formaldehyde solution. The tissues of mice in blank, PVAX1-IRES (NC), PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 (HPV16), PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L (HPV18) and combination of PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 and PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L (HPV16+HPV18) group were then dehydrated, paraffin-embedded and sliced (4 μm thick), and then they were observed by optical microscope.

splenic lymphocyte was investigated by CTL. As shown in **Figure 9F** and **9G**, there was no significant difference of the killing activity of splenic lymphocyte between blank and PVAX1-IRES (NC) group, while the killing activity of splenic lymphocyte was significantly increased in HPV16, HPV18 and HPV16+HPV18 groups compared to NC group. The results showed that GM-CSF and Flt3L enhanced the killing effect on tumors of HPV16/18 vaccine.

Discussion

Two early proteins E6 and E7 play a key role in the malignant transformation of HPV16 and HPV18. E7 can make cell cycle out of control by interacting with Rb family [20]. E6 can prevent DNA repair or programms cell death by binding and degrading p53 [21]. The late protein L1 has the characteristics of self-assembling virus like particles (VLP). L2 could assemble L1 into VLP and stabilize capsid structure of the virus. Some studies have proved that the fusion of E7, L1 and L2 can increase the molecular weight and antigenicity of antigen, which plays a therapeutic effect [22]. In this experiment, E6 and E7 fragments were linked to L1 and L2 to form chimeric proteins and reduce the effect of protein recombination on the immunity of L1 and L2 protein.

The most important role of a successful vaccine is to facilitate the immune response of the organism occurring rapidly and strongly upon a second exposure to the same antigen [23]. In this study, the specific antigen immune response was tested by ELISA. IFN-γ is mainly a cytokine secreted by Th1. The Th1 bias mainly mediates the immune response and it is evidenced by elevated cytokine levels produced by splenocytes or in serum [23]. It can be found that these two vaccines could induce the activation of immune response in the current study.

The combination of Flt3L and Flt3R expressed on immature DCs can selectively expand the number of DCs precursor cells, and promote the maturation of DCs as well. Flt3R is one of the most important cytokines stimulating the generation of DCs *in vivo and in vitro* [24]. In the immune response induced by GM-CSF-B.7.1 gene vaccine, the co-expressed nucleic acid

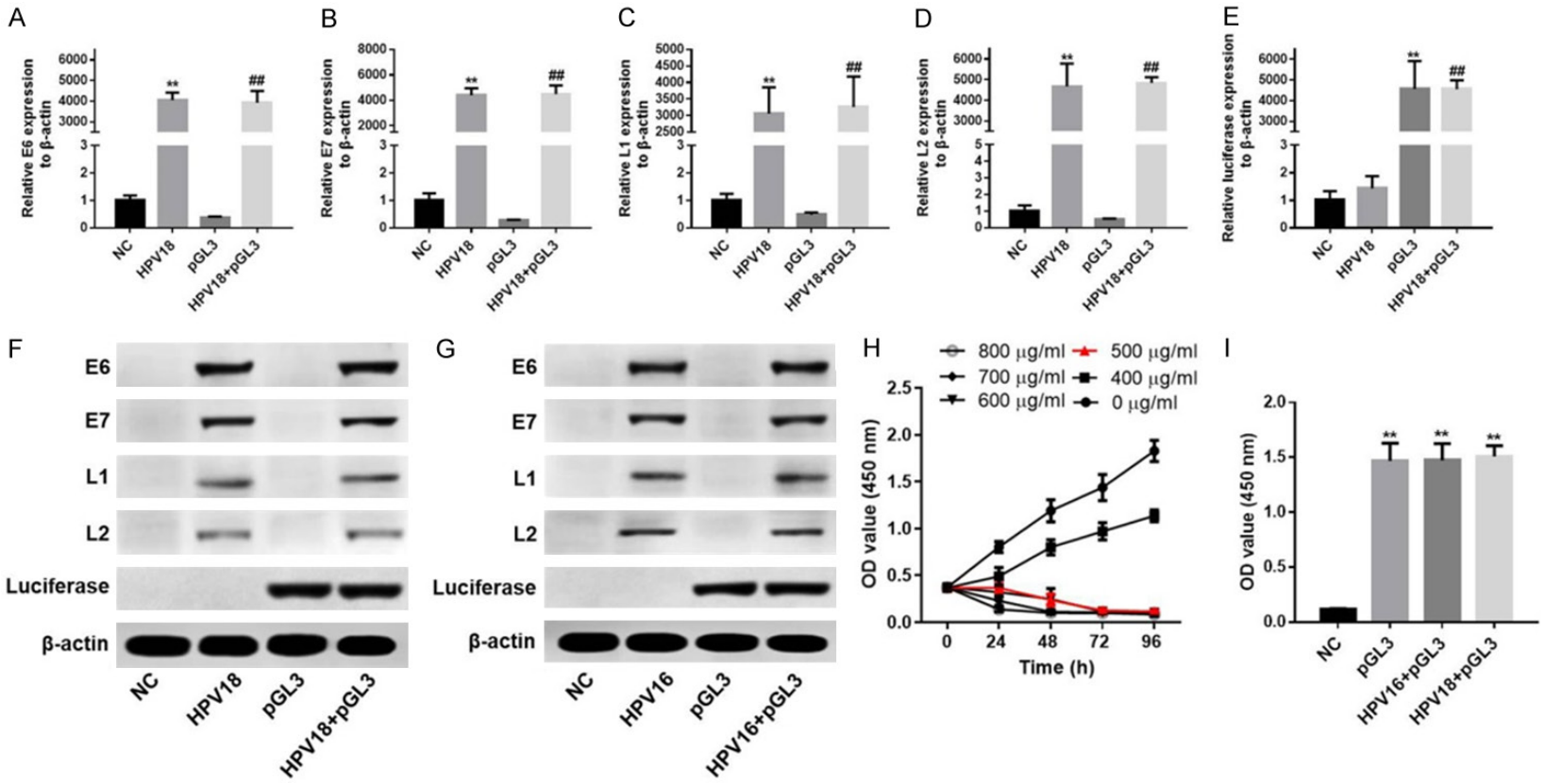

Figure 7. The stable transfections were successfully constructed. Overexpression of E6 (A), E7 (B), L1 (C), L2 (D) and Lucifercase (E) was verified by qRT-PCR. Western blot was used to verify the protein overexpression of E6 E7 L1 L2 and lucifercase in stable transfections of pIRES-neo3-HPV18 E6 E7 L1 L2 (F), pIRES-neo3-HPV16 E6 E7 L1 L2 (G). B16 cells were treated with G418 at concentrations of 0, 400, 500, 600, 700 and 800 μg/ml, and their proliferation activity was measured by MTT at 24, 48, 72 and 96 h (H). B16 cells transfected with pIRES-neo3 and pIRES-neo3-HPV16 E6 E7 vectors were treated with G418 at 500 μg/ml. After 72 h of incubation, the cell proliferation activity was detected (I). $^{**}P < 0.01$ vs. NC group, $^{\#\#}P < 0.01$ vs. pGL3 group.

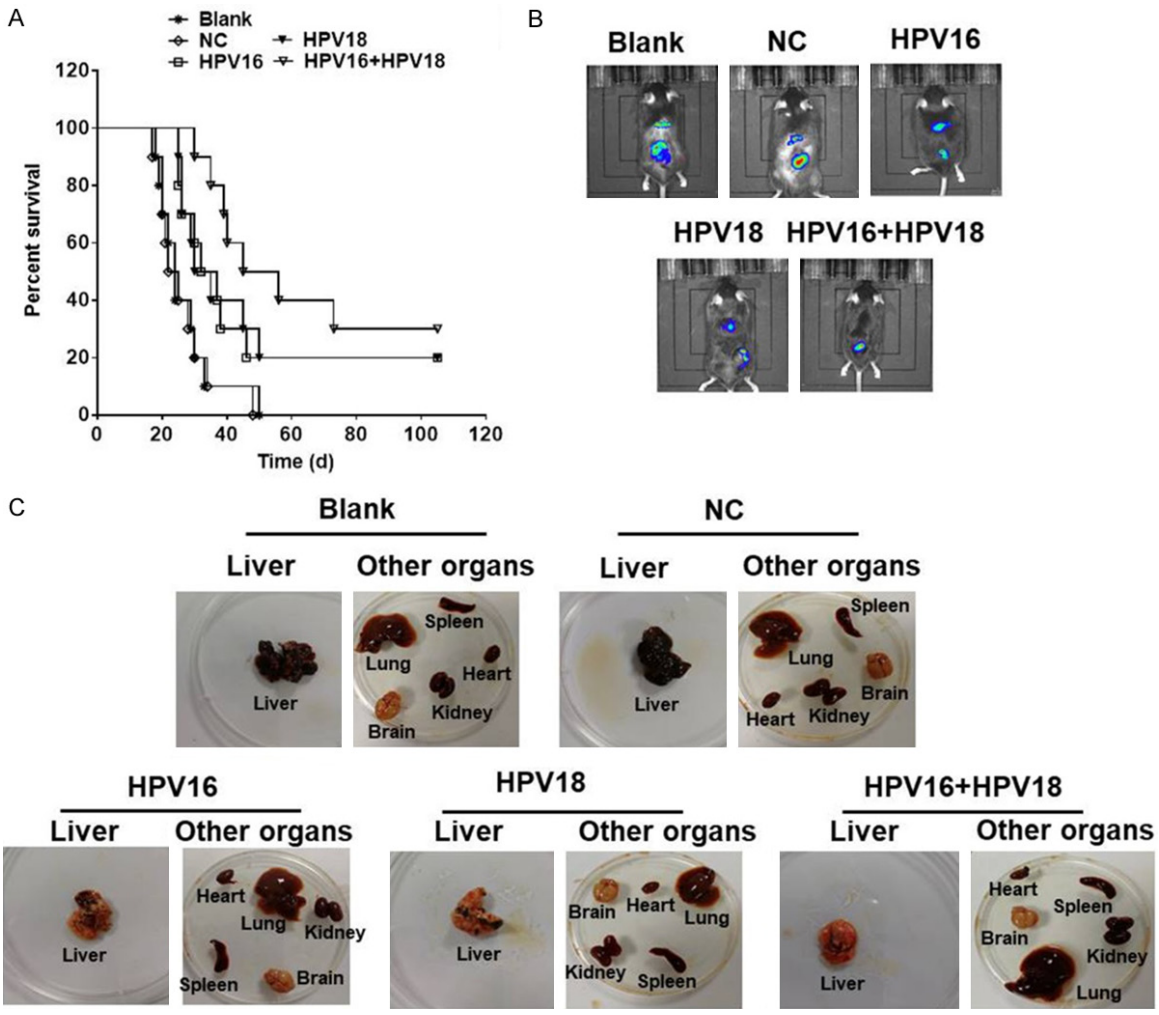

Figure 8. Nucleic acid vaccine prevents proliferation and metastasis of tumor *in vivo*. A. The survival rate of mice in blank, PVAX1-IRES (NC), PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 (HPV16), PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L (HPV18) and their combination (HPV16+HPV18) group at different time point were calculated. B. The size and metastasis of tumor in mice were detected by IVIS. C. Mice were injected with HPV16 E6 E7+Luc stable cells in each treatment group, and then they were sacrificed for observation of the metastasis of other organs (heart, liver, spleen, lung, kidney and brain tissue) after 4 weeks.

vaccine elicits a higher level of antibody and CTL response, thus playing a protective role. The researchers cloned the genes encoding GM-CSF and MAGE-1 into the same plasmid and activated them with different promoters [25]. Immunization with B16-MAGE-1 melanoma yielded a higher IgG antigen-specific response than with MAGE-1 or GM-CSF alone. In this study, eukaryotic expression plasmids PVAX1/Flt3-L and PVAX1/GM-CSF-B.7.1 were constructed and used as gene adjuvants for PVAX1/HPV16 E6 E7 L1 L2 and PVAX1/HPV18 E6 E7 L1 L2 vaccines. It was found that the combination of the recombinant plasmids of these two cytokines could significantly reduce the tumorigenesis rate of mice via enhancing the killing activity of specific CTL . It was also found that the tumorigenic rate of mice treated with the combination (PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 and PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L) vaccine decreased more than that of mice treated with PVAX1-HPV16 E6 E7 L1 L2-IRES-GM-CSF-B.7.1 or PVAX1-HPV18 E6 E7 L1 L2-IRES-Flt3-L. This outcome may due to the non-specific and anti-tumorigenic effect of cytokines Flt3L and GM-CSF. Despite the fact that there was no way to go to the mechanism deeply, this study still provided us a new potential method for treating cervical cancer.

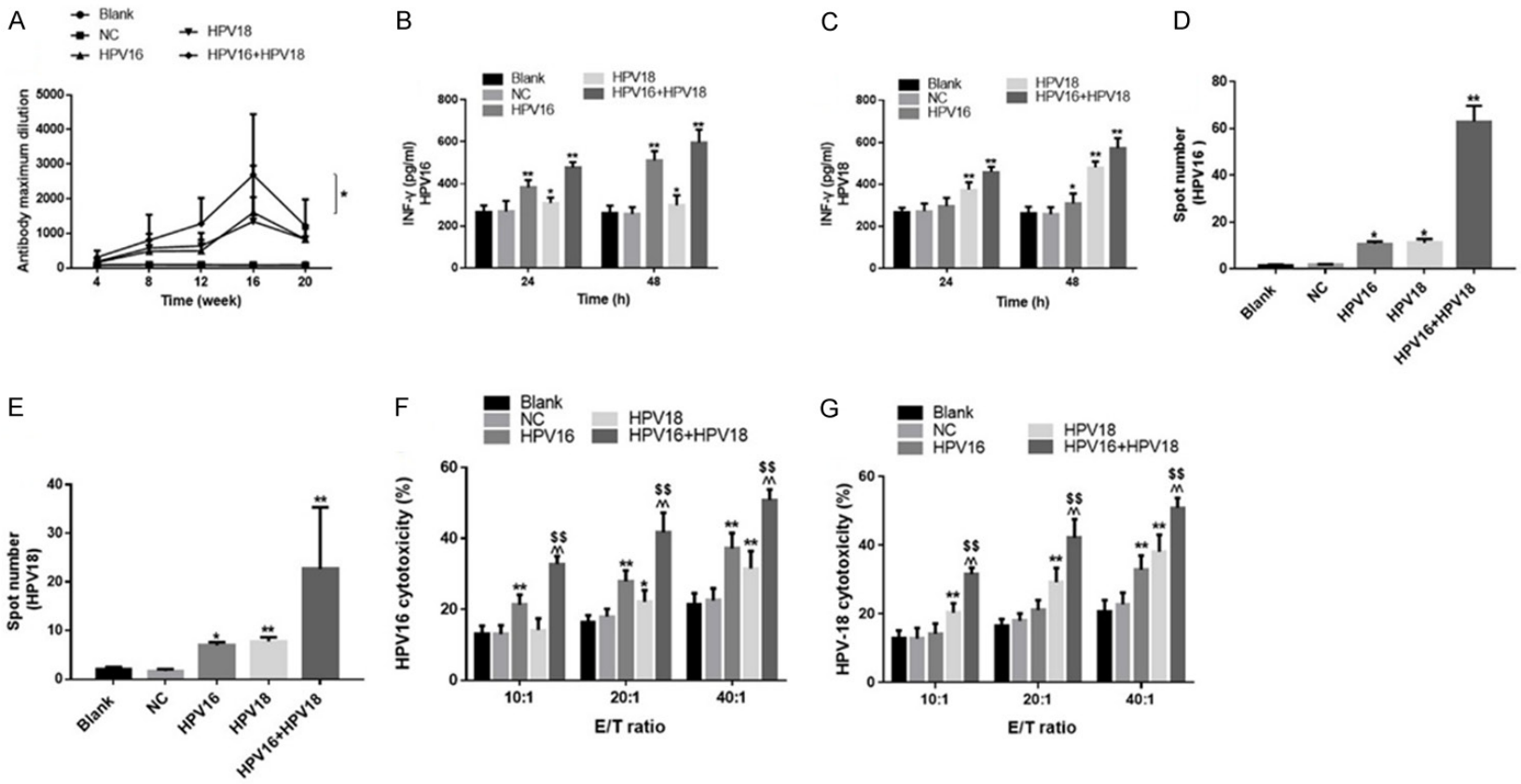

Figure 9. Nucleic acid vaccine has significant immune effect and tumor killing effect. (A) The titer of mice serum specific antibodies was detected by ELISA after 4, 8, 12, 16, and 20 weeks. (B, C) The levels of INF-γ in the culture supernatant of splenic lymph nodes in stable transfections of pIRES-neo3-HPV16 E6 E7 L1 L2 (B) and pIRES-neo3-HPV18 E6 E7 L1 L2 (C) were detected by ELISA. (D, E) ELISpot was used to detect the number of splenic secreting INF-γ in stable transfections of pIRES-neo3-HPV16 E6 E7 L1 L2 (D) and pIRES-neo3-HPV18 E6 E7 L1 L2 (E). (F, G) The killing activity of PVAX1-HPV16 E6, E7, L1, L2-IRES-GM-CSF-B.7.1 (HPV16) (F) and PVAX1-HPV18 E6, E7, L1, L2-IRES-Flt3-L (HPV18) (G) at 10:1, 20:1 and 40:1 ratio was tested by CTL. $^{*}P < 0.05$ vs. NC group, $^{**}P < 0.01$ vs. NC group, $^{\wedge\wedge}P < 0.01$ vs. HPV16 group, $^{\$\$}P < 0.01$ vs. HPV18 group.

In conclusion, this study showed that Flt3L and GM-CSF can significantly improve the immune response and anti-tumor effect of HPV16 E6 E7 L1 L2 and HPV18 E6 E7 L1 L2 vaccines, and these findings may provide an important experimental basis for cancer treatment.

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Disclosure of conflict of interest

None.

Address correspondence to: Yunsheng Xu, Department of Dermatovenereology, The Seventh Affiliated Hospital, Sun Yat-Sen University, No. 628 Zhenyuan Road, Shenzhen 518107, Guangdong, China. E-mail: xuyunsheng1018@163.com; Rongying Ou, Department of Gynaecology and Obstetrics, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, China. E-mail: ourongying@163.com

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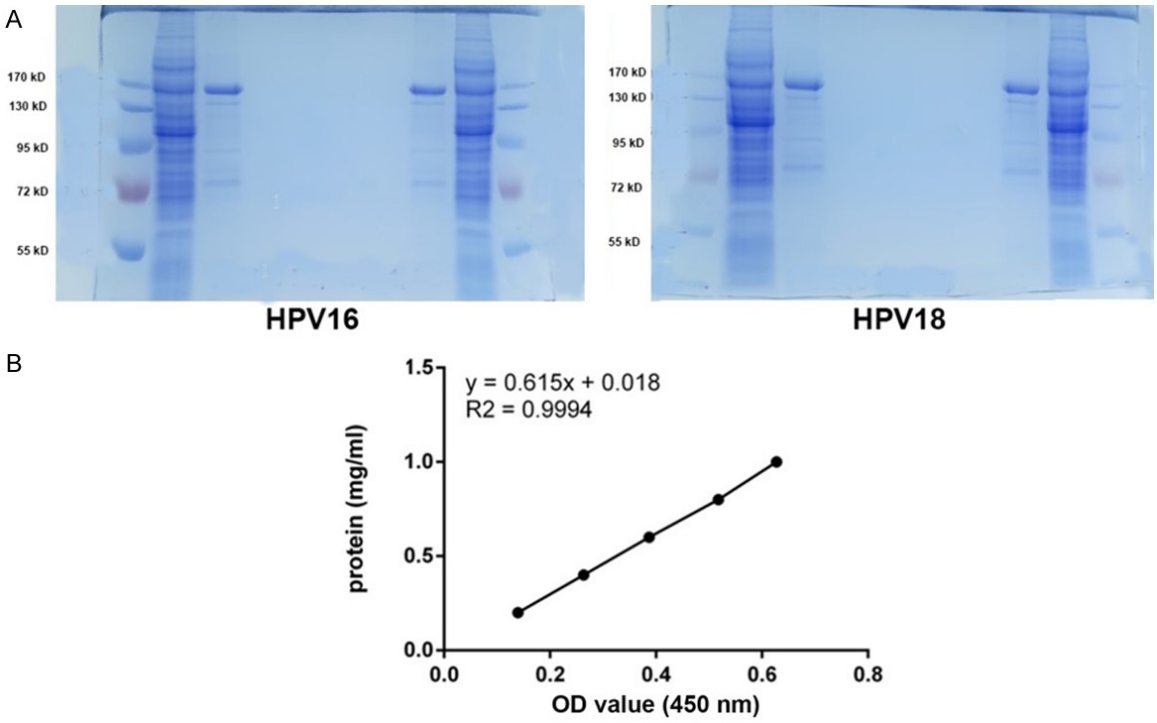

Supplementary Figure 1. The purified HPV16 E6 E7 L1 L2 and HPV18 E6 E7 L1 L2 proteins were successfully obtained. A. The gel map of SDS-PAGE before and after protein purification. B. The standard curve of purified protein was quantified by using BCA.