ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
October 2021; 20(5):525-536.
Doi: 10.18502/ijaai.v20i5.7403

Designing of DNA Vaccine Based on a Secretory Form of Major Capsid Protein of Human Papillomavirus Type 18

Somayeh Shokri[1,2], Shahab Mahmoudvand[1,2], Manoochehr Makvandi[1,2], Reza Taherkhani[3], Mohammad Rashno[4], Farid Azizi Jalilian[5], Kambiz Ahmadi Angali[6], and Mohammad Ali Foroughi[7]

[1] Infectious and Tropical Disease Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2] Department of Virology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3] Department of Virology, School of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran
[4] Department of Immunology, Medicine Faculty, Cellular and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[5] Department of Virology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
[6] Department of Biostatistics, School of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[7] Department of Pathobiology, Section Biotechnology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad Iran

Received: 10 January 2021; Received in revised form: 12 May 2021; Accepted: 22 May 2021

ABSTRACT

More than 99% of cervical cancers are associated with human papillomaviruses (HPVs) worldwide. Current HPV vaccines are safe, highly immunogenic, with effective immunity against specific HPV types. However, DNA vaccines are a new appealing platform which can be considered for designing the HPV vaccines. This study aimed to construct a recombinant eukaryotic expression plasmid containing *L1* of *HPV-18*, tissue plasminogen activators *(tPA)*, and pan HLA DR-binding epitope *(PADRE)* genes into the pVAX1 vector.

The *L1*, *tPA*, and *PADRE* genes were amplified in a thermocycler. The polymerase chain reaction (PCR) products were cloned and insertion of the genes was confirmed using colony PCR, restriction enzymes analysis, and sequencing methods. Indirect immunofluorescence, RT-PCR, and western blot assays were applied to identify the target gene in HEK-293 cells. Total IgG and its isotypes in immunized mice were measured by enzyme-linked immunosorbent assay technique.

Western blot analysis showed a protein band of about 67.5 kDa in supernatant and cell lysate of transfected cells. The results of mice immunization with different constructs (group 1: the pVAX-L1, group 2: pVAX-tPA-PADRE-L1, group 3: pVAX1, and group 4: PBS as controls) indicated that the pVAX1-tPA-PADRE-L1 construct induced a significantly higher level of total IgG than pVAX1-L1 (p=0.003).

In conclusion, pVAX1-tPA-PADRE-L1 recombinant plasmid is a highly immunogenic

Corresponding Author: Manoochehr Makvandi, PhD; Infectious and Tropical Disease Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Tel: (+98 916) 6181 683, Fax: (+98-61) 1373 8313, E-mail: manoochehrmakvandi299@gmail.com

construct and suggests as a promising candidate for vaccine development against HPV type 18 in low-middle-income countries.

Keywords: Alphapapillomaviruses; DNA vaccines; Indirect fluorescent antibody technique; Polymerase chain reaction; Uterine cervical neoplasms; Western blotting

INTRODUCTION

Globally, infectious agents account for almost one-fifth of human cancers. It is estimated that 15–20% of all human cancers may be caused by oncogenic viruses worldwide.[1,2] Persistent infection by Human papillomavirus (HPV) is the important factor for the development of several human cancers, including cervical cancer, vulvar cancer, vaginal cancer, anal cancer, penis cancer, a subset of head and neck cancers (HNCs), colorectal cancer and particularly oropharyngeal cancer.[3,4] In less developed regions cervical cancer is the second most common cancer in women, with an estimated 570,000 new cases and nearly 311,000 deaths every year.[5] More than 200 different HPV genotypes have been identified, which are classified into low-risk (LR) and high-risk (HR) types.[6] Among the HR types, HPV-16 is the most common type has been associated with 50% of all cervical cancers, HPV-18 accounts for 10–15%, and HPV types 31, 33, 45, 52, and 58 accounts for 2–5% cervical cancers. Therefore, HPV-16 and 18 together account for approximately 70% of cervical cancers worldwide.[7]

HPV immunization with three commercial virus-like particles (VLPs) based vaccines, Gardasil, Gardasil 9, and Cervarix, by induction of neutralizing antibody response, can prevent up to 70% of cases of cervical cancer as well as 90% of genital warts due to HPV infections.[8] A successful immunization program primarily depends on the stability, efficacy, safety, and potency of the vaccines.[9] These preventive HPV vaccines have excellent safety and are highly immunogenic. The ability of VLPs to target dendritic cells (DCs) is an important advantage of VLP vaccines and the presentation of antigens into major histocompatibility complex (MHC) class I or class II molecules help B cells to produce antibodies and enhance $CD8^+$ T cells cytotoxicity.[10] low-and middle-income countries (LMICs) carry the greatest burden of HPV infections and the high cost of VLP vaccines remains a problem in this countries.[11]

DNA vaccines have a simple design and do not need cell culture, inactivation of infectious pathogens, and a cold chain. Due to producing in the bacterial cells on the large scale are relatively inexpensive and easy to manufacture and cost-effective for LMICs.[12,13] DNA vaccines when injected intramuscularly or intradermally,[14] the antigen can be presented by both major histocompatibility complex class I and class II complexes thereby enable the activation of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells in addition to B cells. Fusion of the target antigen to adjuvants that enhance antigen-targeting strategy, improve immunological responses.[15]

Herein; we designed a DNA plasmid vaccine encoding the *tPA-PADRE*-full length *L1* gene against HPV-18. To this end, we used from pVAX1 vector containing cytomegalovirus (CMV) and T7 promoters which have been approved by the Food and Drug Administration (FDA) in a clinical trial in the development of DNA vaccines.

MATERIALS AND METHODS

Amplification of Full *L1* Gene by Polymerase Chain Reaction (PCR)

The optimized HPV-18 *L1* gene was kindly provided by Razi Vaccine and Serum Research Institute, Karaj, Iran. Forward primer (Bioneer, Korea)with a restriction site (underlined) for the SalI 5'-AGCATCGTCGACTGTTTGTATACTAGGGTTCTT ATCCTTC-3', and reverse primer (Bioneer, Korea) with two stop codon sequence and contains a restriction site (underlined) for XhoI 5'- AAG CTC GAG TTA TTA TTT TCT TGC CCT TAC ACG A-3'. The PCR reaction mixture containing 1 µL (200 ng/µL) extracted DNA, 2µL from each primer (10 µM), 25 µL master mix (Sinaclon, Iran), and D/W up to 50 µL. The mixture was subjected to Thermocycler (PEQLAB, Germany) with the following conditions: 95°C for 5 min followed by 30 cycles at 94°C for 50 sec, at 55°C for 60 sec, at 72°C for 120 sec and 72°C for 300 sec. The PCR product of the *L1* gene (1728 bp) was

extracted from 1.2% agarose gel using Gel/PCR purification Kit (YektaTajhiz, Iran) according to the manufacturer's protocol.

Amplification of *tPA-PADRE* Gene by PCR Reaction

The *tPA-PADRE* synthetic gene included a Kozak sequence and a restriction site for the NheI[16] was amplified; using the forward T7 primer 5'-TAA TAC GAC TCA CTA TAG GG-, and reverse primer contained a restriction site (underlined) for HindIII with 5'-ATT ACG AAG CTT GTC GAC AGC AGC CGC CTT CAG GGT CCA AGC A-3'. The PCR reaction mixture containing the 1 µL (115 ng/µL) extracted DNA, 2 µL from each primer (10 µM), 25 µL master mix (Sinaclon, Iran), and D/W up to 50 µL. The mixture was subjected to Thermocycler under the following conditions: 95°C for 5 min followed by 30 cycles at 94°C for 30 sec, at 55°C, at 72°C for 30 sec and 72°C for 300 sec. The PCR product of the *tPA-PADRE* gene (174 bp) was extracted from 2% agarose.

Cloning and Construction of the Recombinant Plasmid pVAX1-tPA-PADRE

A total of 41.2 µL (3 µg/mL) of the amplified *tPA-PADRE* gene was digested in a reaction mixture containing 1 unit of NheI and HindIII (Thermo Fisher Scientific, USA), 5 µL of the Tango buffer (10 X) and D/W up to 50 µL and kept at 37°C overnight. To construct pVAX1-tPA-PADRE, the digested *tPA-PADRE* gene was ligated into pVAX1 (2999 bp, 153 ng/µL) digested with the same enzymes. The 10 µL volume ligation reaction mixture containing; 0.2 µL (14.55 ng/µL) of the gene, 0.5 µL (50 ng/µL) vector, 1 µL of 10X ligation buffer, 7.3 µL of D/W, and 1 µL of T4 DNA ligase (200 U/L) (Thermo Fisher Scientific, USA) was incubated at 22°C for 1 hour. The ligation mixture was transformed into competent cells (E. coli, DH5α strain) and dispensed on an agar plate containing 50 µg/mL of kanamycin (Sigma, USA) and was incubated at 37°C for 16 hours. Several colonies were selected and assayed by colony PCR (PCR product: 291 bp) with T7 forward and bovine growth hormone (BGH, TAGAAGGCACAGTCGAGG) reverse primers (Bioneer, Korea). After selecting the positive recombinant colonies, the recombinant tPA-PADRE plasmid was extracted from the overnight culture by Plasmid extraction Kit (YektaTajhiz, Iran) according to the manufacturer's protocol. The recombinant plasmids were confirmed by restriction digestion by using NheI and HindIII enzymes.

Cloning and Construction of the Recombinant Plasmid pVAX1-tPA-PADRE-L1

27.3 µL (3 µg/mL) of pVAX1-tPA-PADRE and 34.3 µL (3 µg/mL) of *L1* gene were separately digested by SalI and 3XhoI (Thermo Fisher Scientific, USA) restriction enzymes and ligation reaction was prepared in a 10 µL reaction mixture containing; 2.3 µL (138.8 ng/µL) of L1, 0.5 µL (50 ng/µL) of pVAX1-tPA-PADRE vector, 1 µL of 10X ligation buffer, 5.2 µL of D/W and 1 µL of T4 DNA ligase. The incubation and transformation procedures were carried out as described previously. Several colonies were selected and assayed by colony PCR (PCR product: 1924 bp)with T7 forward and BGH reverse primers.[17] The recombinant plasmids were confirmed by restriction digestion by using SalI and XhoI enzymes and the sequencing of L1 was carried out (Bioneer, Korea).

***In vitro* HPV-18 L1 Protein Expression Cell Culture**

Human embryonic kidney cell line 293 (HEK-293) were expanded in Dulbecco's modified Eagle's medium (DMEM, BioIdea, Iran) supplemented with 10% heat-inactivated fetal bovine serum (FBS, BioIdea, Iran), 1% penicillin, and streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) in 25 cm2 flask (SPL Life Sciences, Korea) at 37°C in 5% CO2. When the cell density reached 75– 80% confluency (20 $x10^5$), the cells were seeded ($3x10^5$) in a 6-well plate (SPL Life Sciences, Korea). Again when the cell density reached 75–80% confluency, the cells were washed twice with phosphate buffer saline (PBS, BioIdea, Iran) and were added Opti-MEM (BioIdea, Iran) without antibiotics to the monolayer of cells in each well. Plasmid transformation was carried out using lipofectamine 2000 (Invitrogen, Carlsbad, CA). The pVAX1-tPA-PADRE-L1 DNA (2 µg) and Lipofectamine reagent (10 µL) were mixed in 500 µL Opti-MEM medium and incubated for 30 min at room temperature. Then 100 µL of the mixture was added to the culture medium. Two wells were considered as a control group.

Indirect Immunofluorescence

Briefly, the transfected cells were fixed and permeabilized with 1 mL ice-cold methanol/acetone

(1:1) for 15 min at -20^{0}C. *L1* expression was detected (Abcam, USA) in dilution of 1:2500 followed by staining with 1 mL fluorescein isothiocyanate (FITC) labeled anti-mouse IgG (CMG, Iran) in dilution of 1:4000. Next, 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, Roche, Germany) was used for staining the nuclei of the cells. After rinsing with ice-cold PBS, coverslips were immediately observed under a fluorescence microscope (HundWetzlar, Germany). The *pVAX1* transfected *HEK-293* cells were used as the control group.

Detection of L1 mRNA by RT-PCR

RNA was harvested from HEK 293T cells; using a total RNA extraction Kit for cell culture (YektaTajhiz, Iran). Complementary DNA (cDNA) was synthesized according to the manufacturer's instructions kit (YektaTajhiz, Iran). HPV-18 *L1* gene was amplified by universal primers MY09 and MY11. The PCR reaction mixture containing 2 µL (150 ng) of cDNA, 0.5 µL from each primer (10 µM), 12.5 µL master mix (Sinaclon, Iran), and D/W up to 25 µL. The mixture was subjected to Thermocycler under the following conditions: 95°C for 5 min followed by 35 cycles at 94°C for 30 sec, at 55°C, at 72°C for 30 sec and 72°C for 300 sec. The PCR product (450 bp) was analyzed by 2% agarose gel electrophoresis. The GAPDH with a PCR product of 496 bp was used as an internal control.

HPV L1 Protein Expression Assay by Western Blotting

For detection of L1 protein expression after transfection, cells were harvested after 72 h and washed with PBS, and then lysed in 300 µL radioimmunoprecipitation assay buffer (RIPA) (CMG, Iran) with 1% protease and phosphatase inhibitor cocktail (CMG, Iran). The proteins were heated in loading buffer 2X (4% SDS/ 20% glycerol/0.004% bromophenol blue/0.125M Tris-Cl pH 6.8/10% 2-mercaptoethanol) at 100°C for 5 min. Total proteins (30 µg per lane) were separated by using 12% sodium dodecyl sulfate-polyacrylamide gels and Western blotting onto PVDF membrane (CMG, Iran) using semi-dry protein transfer apparatus (Bio-Rad, USA). The blotted PVDF membranes were blocked and then were incubated with the mouse HPV18 monoclonal antibody (Abcam, USA) in dilution of 1:2500. The membranes were incubated with a peroxidase-coupled goat anti-mouse antibody (CMG, Iran) in dilution of 1:5000. Detected L1 protein was visualized using the ECL Western blotting kit (CMG, Iran), according to the manufacturer's instructions. Protein concentration was determined by the Bradford method.[18]

***In vivo* Measuring Antibody Response Ethics Statement**

Inbred female BALB/c mice (8 weeks old) were purchased from the Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). All animal procedures were performed according to the approved protocols and following recommendations for the proper use and care of the Animal Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences. The approval No. IR.AJUMS.ABHC.REC.1397.026.

Immunizations

Four groups, each consisted of ten female BALB/c mice. One group was immunized with pVAX-tPA-PADRE-L1, the second group was immunized with pVAX-L1, the third group was immunized with pVAX1, and the fourth group with PBS. Each group of mice was immunized intramuscularly on days 0, 21, and 42 days[19] with 100 µg of plasmid DNA in 100 µL of PBS. Normal control mice were immunized with 100 µL of PBS. Serum samples were then collected at 0, 21, 42, and 56 days after the first immunization. The total IgG and subclasses of IgG (IgG1, IgG2a, IgG2b) antibodies were evaluated in serum samples on day 56 by enzyme-linked immunosorbent assay (ELISA).

Indirect ELISA Assay Optimization

The ELISA was optimized by examining different antigen concentrations and serum dilutions[20] Briefly, ELISA plates (Jet Biofil, Guangzhou, China) were coated with 50 µL/well of serial dilution of L1 HPV 18 (500 ng-1 µg) in 50 mM carbonate buffer (pH 9.6) at 4^{o}C overnight. On the next day, plates were washed with TBS/0.05% Tween 20 and blocked with TBS/1% BSA at 4^{o}C overnight. After washing, the sera from the individual groups were pooled and were diluted (1:20 to 1:100) in TBS/0.05% Tween 20/1% BSA for 2 h at room temperature. HRP-conjugated goat anti-mouse IgG (CMG, Iran) at 1:5000 (to measure total IgG) and 1:5000 (To measure IgG isotypes) dilution in TBS/0.1% Tween 20/1% BSA and the plates incubated for 1 h at room temperature. After incubation for

30 min at room temperature with 3, 3, 5, 5-Tetramethylbenzidine (TMB), the reaction was stopped by the addition of 0.5 M sulfuric acid. The absorbance was measured at 450 nm with a microplate reader (Tecan Trading AG, Switzerland).

Measuring Antibody Response by ELISA

For measurement of the IgG total and subclass, 96-well plates were coated with the L1 protein at 800 ng/well. The sera were added to each coated well from the individual groups at1:80 dilution. HRP-conjugated goat anti-mouse IgG was added subsequently at 1: 5000 (to measure total IgG) and 1:5000 (To measure IgG isotypes) dilution. The absorbance was measured at 450 nm with a microplate reader.

Statistical Analysis

The differences in the data between all the groups were compared by t-test and one-way ANOVA tests. *p* values <0.05 were considered significant.

RESULTS

Integration of *tPA-PADRE- L1* Genes in the pVAX1 was Confirmed by PCR

The PCR products for the *L1* and *tPA-PADRE genes* were 1729 bp (Figure 1A) and 174 bp (Figure 1B), respectively. The constructed plasmids containing pVAX1-tPA-PADRE and pVAX1-tPA-PADRE-L1 were confirmed by colony PCR (Figure 1C and 1D) and restriction enzyme digestion (Figure 1E and 1F). The result of the sequence analysis using SnapGene software showed the *L1* gene sequence was 100% identical to desired gene (MT-450424).

pVAX1-tPA-PADRE-L1 was Successfully Expressed in HEK293

The result of the transfected HEK293 cells with pVAX1-tPA-PADRE-L1 and pVAX1 plasmid (as negative control) by Indirect Immunofluorescence test is shown in Figure 2.

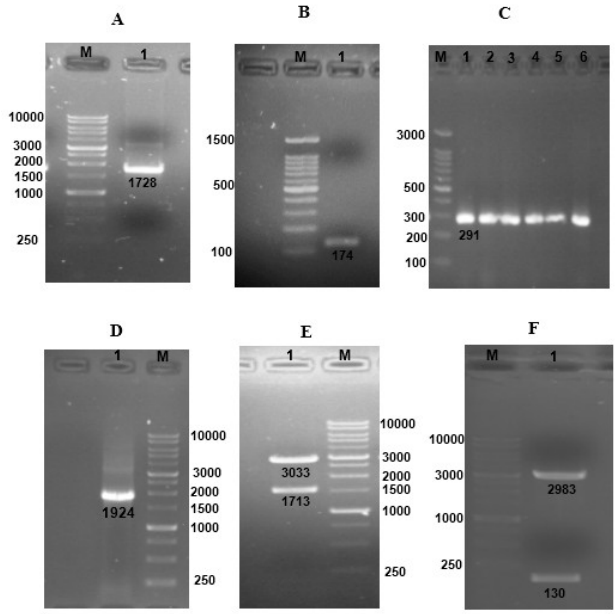

Figure1. (A): Agarose gel electrophoresis of the *L1* gene product. Lane M; 1Kb DNA size marker, Lane 1; a 1728 bp PCR product (B): Agarose gel electrophoresis of the *tPA-PADRE* gene product. Lane M; 100 bp DNA size marker, Lane 1; a 174 bp PCR product (C): Agarose gel electrophoresis of pVAX1-tPA-PADRE due to colony PCR assay with T7 and BGH universal primers. Lane M; 100bp DNA size marker. Lane 1-6; 291bp PCR product (D): Agarose gel electrophoresis of pVAX1-tPA-PADRE-L1 due to colony PCR assay with T7 and BGH universal primers. Lane M; 1kb DNA size marker, Lane 1; a 1924 bp PCR product E: Double digestion of recombinant vector. Lane M; 1kb DNA size marker, Lane 1; pVAX1-tPA-PADRE-L1 plasmids digested by SalI and XhoI (F): Double digestion of recombinant vector. Lane M; 1kb DNA size marker, Lane 1; pVAX1-tPA-PADRE plasmids digested by HindIII and NheI

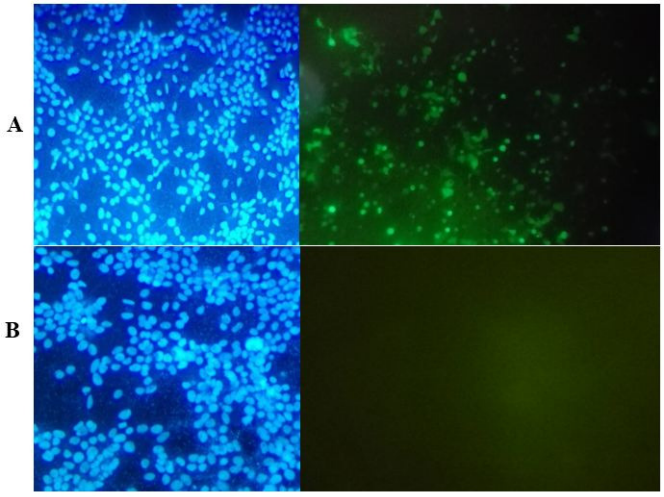

Figure 2. Detection of L1 protein in HEK293 Cells by Indirect Fluorescent Antibody Test. (A) The cells were transfected with pVAX1-tPA-PADRE-L1 (Right) and was stained with diamidino-2-phenylindole (DAPI) (Left). (B) The cells were transfected with pVAX1 (Right) and were stained with DAPI (Left).

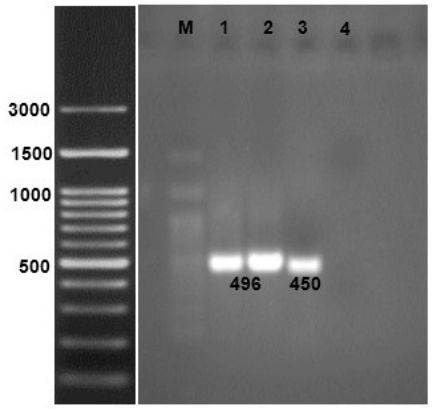

Figure 3. Detection of L1 mRNA in transfected HEK-293 cells by Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Lane M: molecular marker (100 bp), Lane 1: *GAPDH* gene with a size of 496 bp in cDNA cells extracted that transfected with pVAX1; Lane 2: *GAPDH* gene with a size of 496 bp in cDNA cells extracted transfected with pVAX1-tPA-PADRE-L1; lanes 3, positive band with a size of 450 bp in transfected cells with expression plasmids pVAX1-tPA-PADRE-L1, Lane 4: negative results in transfected cells with pVAX1 plasmid.

L1 Gene Expression was Confirmed in the mRNA and Protein Levels

The L1 mRNA expression was confirmed by RT-PCR in transfected cells and is shown in Figure 3.

A protein band of approximately 67.5 kDa molecular weight has confirmed the presence of HPV major capsid protein L1. Western blot analysis has detected the expression of the tPA-PADRE-L1 protein in both the supernatant and lysates collected from the transfected HEK293 cells (Figure 4).

Humoral Response of Vaccinated Mice

Each of the 20 mice immunized with pVAX-tPA-PADRE-L1 and pVAX-L1 had generated positive ELISA titers to Sera. As shown in Figure 5, mice immunized with the tPA-PADRE-L1 constructs induced a significantly higher level of total IgG than pVAX-L1 immunization did (p=0.003).

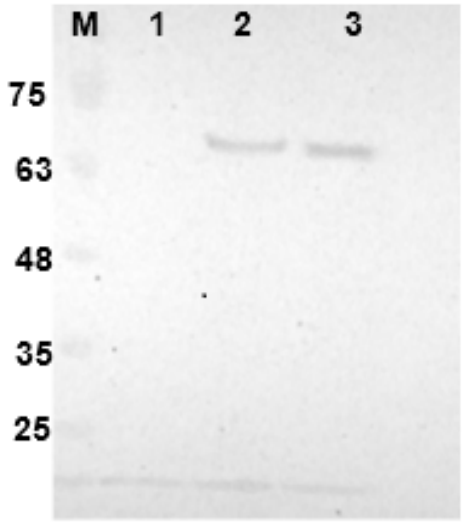

Figure. 4. The L1 protein band is seen at about 67.5 kDa. Lane M, the protein marker; Lane 1, transfected 293 cells with pVAX1 as negative control; Lane 2, supernatant of HEK293 cells transfected with pVAX1-tPA-PADRE-L1 recombinant plasmid; Lane 3, cell lysate of transfected 293 cells with pVAX1-tPA-PADRE-L1 recombinant plasmid.

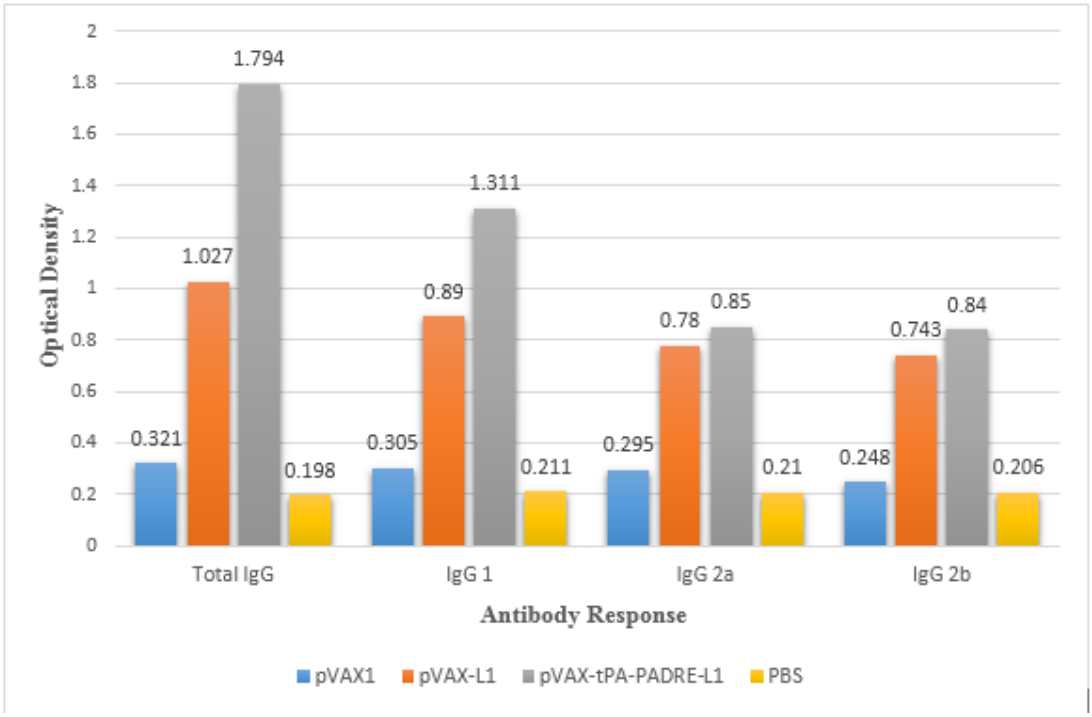

Figure 5. Humoral response in mice after immunization with the pVAX1-tPA-PADRE-L1 and pVAX1-L1 recombinant plasmids

DISCUSSION

Most of the populations for both women and men showed a large peak of HPV incidence during adolescence after the onset of sexual activity.[21] Due to the lack of effective treatment, it would be wise to utilize an effective vaccine that is capable of protecting against at least HPV types 16 and 18 to prevent cervical cancer plus other HPV-associated cancers.[22] Unfortunately, VLP vaccines are very expensive and unaffordable for most people in developing countries.[23] DNA vaccines have great potential for prevention due to their stability, safety, simplicity of manufacturability, low cost of production, and ability to induce antigen-specific immunity.[24] Enhancing the immune response and induction of both humoral and cell-mediated immunity against vaccine antigens strongly depend on adjuvant formulations.[15] Immunologic adjuvants help to increase the efficacy of weak antigens when used in combination with specific vaccine antigens.[25]

One strategy for the enhancement of DNA vaccine potency is the induction of $CD4^+$ T helper cells.[26] Interaction between B and $CD4^+$ T cells is crucial for their optimal responses in adaptive immunity.[27,28] The pan HLA DR-binding epitope (PADRE) is one of the most effective molecules used to induce $CD4^+$ responses for the development of synthetic and recombinant vaccines.[29] PADRE can enhance the potency of vaccines in preclinical models, as well as mouse models and can also bind to a wide variety of MHC-II molecules and induces proliferative human T cells response with more than 16 types of common HLA-DR.[30,31]

Another strategy to enhance the immunogenicity of DNA vaccine efficacy is targeting DNA vaccine to professional antigen-presenting cells (APCs).[32] Professional APCs, especially DCs, play an important role in T cell and B cell functions, including proliferation and antibody isotype switching.[33] The secreted antigen is more efficient along with antigen presentation to migratory DCs for presentation on MHC class I molecules, thus are generally more immunogenic, for both B and T cells.[32,34] Tissue plasminogen activator signal sequence (tPA) leader sequence into DNA constructs can help the secretion of the encoded antigen that could be taken up as an exogenous antigen by APCs and B cells.[35] In Several studies, tPA demonstrated an effective signal peptide for enhancing immune efficacy.[16,36-39]

We designed and constructed a DNA vaccine; using pVAX1 encoding tPA-PADRE-L1 to evaluate its expression in HEK-293 cells. To increase the protein expression level we added the Kozak sequence to the upstream of the initiation codon.[40] Finally the immunogenic of this construct was examined in mice by ELISA assay.

Our data showed that pVAX1-tPA-PADRE-L1 is capable of infecting the HEK 293 cell line *in vitro*. The size of the expressed HPV18 L1 proteins on western blots was67.5 kDa. Our western blot result showed the presence of the L1 protein in supernatant cells. One of the goals of this study was to determine the evaluation of the specific IgG subclasses against L1 HPV 18. The analysis revealed that the IgG1 ($p < 0.001$), IgG2a ($p< 0.001$), and IgG 2b ($p < 0.001$) were nificantly higher in immunized mice with a recombinant pVAX1-tPA-PADRE-L1compared to mice immunized with a recombinant pVAX1-L1. PADRE help to induce $CD4^+$ T cell response to evoke both the humoral and cellular responses where Th1 type cells elicit IgG2a antibody production and Th2 type cells induce IgG1 antibody secretion.[41,42] The predominant isotype subclass of the antibodies generated to the DNA vaccines encoding secreted antigens mostly is IgG1.[32] Surprisingly, IgG1 was the predominant subclass from immunization with pVAX1 vector containing *tPA-PADRE-HPV18 L1* genes. In this study, the level of IgG1 and IgG2a were more than IgG2b in the group immunized with the tPA-PADRE-HPV18 L1 vaccine. The amount of IgG1 and IgG2a antibodies produced in response to tPA-PADRE-HPV18 L1 (IgG1/IgG2a ratio: 1.54) are higher than in response to pVAX-L1 (IgG1/IgG2a ratio: 1.14). This is the first report to determine the evaluation of specific IgG subclasses against a secretory form of L1 HPV 18.

As mentioned above, APCs and $CD4^+$ T cell interactions can enhance the immunogenicity of DNA vaccine efficacy. The contact of $CD4^+$ T cells with DCs leads to the maturation of DCs, which subsequently leads to the activation of $CD8^+$ T cells.[43] Yang et al was demonstrated that the employment of DNA encoding HPV L1 or L2 may lead to the local activation of DCs, resulting in the generation of antigen-specific $CD4^+$ T cells and neutralizing antibodies.[44] In a study was reported the induction of IgG2a and IgG1 antibodies

after oral immunization with HPV-11 VLPs.[45] Similar results were obtained following coadministration of HPV-16 or HPV-18 VLPs with LT R192G adjuvant.[46] Kim et al was observed the highest IgG induction in HPV16 L1 DNA vaccines fused with a secretion signal RANTES. The vaccine-induced higher levels of IgG antibodies with higher levels of IgG2a than IgG1.[47] These discrepancies may be observed for differences in adjuvants, immunization methods, sources of antigens, or antigens dose.

Previous studies have shown that PADRE acts as a potent adjuvant and is capable to enhance vaccine potency.[48,49] Fu Hung et al have reported co-administration of HPV E6 or E7 antigen with DNA encoding Ii-PADRE was able to generate$CD4^+$ T cell immune responses to enhance $CD8^+$ T-cell immune responses.[50] In another study, Wu et al described the design of the Ii-PADRE-E6 DNA vaccine that can lead to enhanced HPV E6 antigen processing and presentation through MHC class I molecules in facilitating the activation and proliferation of antigen-specific $CD8^+$ T cells.[51] Vaccination with the combination of calreticulin (CRT)/E6 DNA with CIITA DNA and Ii-PADRE leads to enhanced E6-specific $CD8^+$ memory T cells in vaccinated mice.[52] Also several reports indicate that DNA vaccine fused TPA can induce significant high levels of IgG antibodies.[53] Apart from HPV DNA vaccine, tPA or PADRE has been used in the design of other DNA vaccines such as Tuberculosis (TB),[54] H5N1,[55] human immunodeficiency virus (HIV),[56] hepatitis E virus (HEV),[16] Japanese encephalitis virus (JEV),[36] and Malaria.[57]

In conclusion, the currently licensed HPV vaccines are well tolerated and showed well humoral immune responses in humans. Unfortunately, the numbers of high-risk group HPV are increasing among the population in low middle-income countries and due to the high cost of the HPV vaccine, it is not affordable for them. Therefore, they need a cost-effective HPV vaccine to eliminate cervical cancer and reduce the high mortality rate. The results of this study show the HPV 18 DNA vaccine promising a novel candidate for vaccine development in low-middle-income countries. This study constitutes a step towards generating an affordable HPV vaccine in future studies. However, the immunogenicity of this recombinant plasmid requires to be evaluated *in vivo* on induction of cell-mediated response in addition to the humoral response as novel DNA vaccines in future investigations. Further study of this vaccine strategy is in progress.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest to declare.

ACKNOWLEDGEMENTS

This study, as a part of my PhD thesis, was performed by Somayeh Shokri. This project was financially supported by the Infectious and Tropical Diseases Research Center of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran (grant No. OG-9720).

REFERENCES

1. McLaughlin-Drubin ME, Munger K. Viruses associated with human cancer. Biochim Biophys Acta. 2008;1782(3):127-50.
2. Shokri S, Mahmoudvand S, Taherkhani R, Farshadpour F, Jalalian FA. Complexity on modulation of NF-κB pathways by hepatitis B and C: A double-edged sword in hepatocarcinogenesis. J Cell Physiol. 2019;234(9):14734-42.
3. Garbuglia AR, Lapa D, Sias C, Capobianchi MR, Del Porto P. The Use of Both Therapeutic and Prophylactic Vaccines in the Therapy of Papillomavirus Disease. Front Immunol. 2020;11(2):188-9.
4. Mahmoudvand S, Safaei A, Erfani N, Sarvari J. Presence of human papillomavirus DNA in colorectal cancer tissues in Shiraz, Southwest Iran. Asian Pac J Cancer Prev. 2015;16(17):7883-7.
5. Rahman R, Clark M, Collins Z, Traore F, Dioukhane E, Thiam H, et al. Cervical cancer screening decentralized policy adaptation: an African rural-context-specific systematic literature review. Glob Health Action. 2019;12(1):1587894.
6. Gheit T. Mucosal and cutaneous human papillomavirus infection and cancer biology. Front Oncol. 2019;9:355.
7. Mahmoudvand S, Shokri S, Makvandi M, Taherkhani R, Rashno M, Jalilian FA, et al. In silico prediction of T-cell and B-cell epitopes of human papillomavirus type 16 L1 protein. Biotechnol Appl Biochem. 2021:1-12.
8. Salina Zhang B, Batur P, Ncmp C. Human papillomavirus in 2019: An update on cervical cancer

prevention and screening guidelines. Cleve Clin J Med. 2019;86(3):173-8.
9. Shokri S, Shahkarami MK, Shafyi A, Mohammadi A, Esna-Ashari F, Hamta A. Evaluation of the thermal stability of live-attenuated Rubella vaccine (Takahashi strain) formulated and lyophilized in different stabilizers. J Virol Methods. 2019;264(5):18-22.
10. Zepeda-Cervantes J, Ramírez-Jarquín JO, Vaca L. Interaction Between Virus-Like Particles (VLPs) and Pattern Recognition Receptors (PRRs) From Dendritic Cells (DCs): Toward Better Engineering of VLPs. Front Immunol. 2020;11(1100).
11. Parkin DM. The global health burden of infection-associated cancers in the year 2002. Int J Cancer. 2006;118(12):3030-44.
12. Rajčáni J, Moško T, Režuchová I. Current developments in viral DNA vaccines: shall they solve the unsolved? Rev Med Virol. 2005;15(5):303-25.
13. Fioretti D, Iurescia S, Fazio VM, Rinaldi M. DNA vaccines: developing new strategies against cancer. J Biomed Biotechnol. 2010;2010:174378.
14. Davis HL. Intramuscular and intradermal injection of DNA vaccines in mice and primates. Methods Mol Med. 2000;29:71-7.
15. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity. 2010;33(4):492-503.
16. Farshadpour F, Makvandi M, Taherkhani R. Design, construction and cloning of truncated ORF2 and tPAsp-PADRE-truncated ORF2 gene cassette from hepatitis E virus in the pVAX1 expression vector. Jundishapur J Microbiol. 2015;8(12):e26035.
17. Farshadpour F, Taherkhani R, Makvandi M, Rajabi Memari H, Samarbafzadeh AR. Codon-Optimized Expression and Purification of Truncated ORF2 Protein of Hepatitis E Virus in Escherichia coli. Jundishapur J Microbiol. 2014;7(7):e11261-e.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248-54.
19. Lladser A, Párraga M, Quevedo L, Molina MC, Silva S, Ferreira A, et al. Naked DNA immunization as an approach to target the generic tumor antigen survivin induces humoral and cellular immune responses in mice. Immunobiology. 2006;211(1-2):11-27.
20. Taherkhani R, Makvandi M, Farshadpour F. Development of enzyme-linked immunosorbent assays using 2 truncated ORF2 proteins for detection of IgG antibodies against hepatitis E virus. Ann Lab Med. 2014;34(2):118-26.
21. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, et al. Global cancer Observatory: cancer today. Lyon, France: international agency for research on cancer. Cancer Today. 2018.
22. Yin F, Wang Y, Chen N, Jiang D, Qiu Y, Wang Y, et al. A novel trivalent HPV 16/18/58 vaccine with anti-HPV 16 and 18 neutralizing antibody responses comparable to those induced by the Gardasil quadrivalent vaccine in rhesus macaque model. Papillomavirus Res. 2017;3(1):85-90.
23. Madrid-Marina V, Torres-Poveda K, López-Toledo G, García-Carrancá A. Advantages and Disadvantages of Current Prophylactic Vaccines Against HPV. Arch Med Res. 2009;40(6):471-7.
24. Cheng MA, Farmer E, Huang C, Lin J, Hung C-F, Wu TC. Therapeutic DNA Vaccines for Human Papillomavirus and Associated Diseases. Hum Gene Ther. 2018;29(9):971-96.
25. Sanina N. Vaccine Adjuvants Derived from Marine Organisms. Biomolecules. 2019;9(8):340.
26. Kim D, Hoory T, Monie A, Ting JP-Y, Hung C-F, Wu TC. Enhancement of DNA vaccine potency through coadministration of CIITA DNA with DNA vaccines via gene gun. J Immunol. 2008;180(10):7019-27.
27. Taherkhani R, Farshadpour F, Makvandi M, Rajabi Memari H, Samarbafzadeh AR, Sharifi N, et al. Cytokine Profiles and Cell Proliferation Responses to Truncated ORF2 Protein in Iranian Patients Recovered from Hepatitis E Infection. J Trop Med. 2015;2015:523560.
28. Taherkhani R, Farshadpour F, Makvandi M. Design and production of a multiepitope construct derived from hepatitis E virus capsid protein. J Med Virol. 2015;87(7):1225-34.
29. Rosa DS, Tzelepis F, Cunha MG, Soares IS, Rodrigues MM. The pan HLA DR-binding epitope improves adjuvant-assisted immunization with a recombinant protein containing a malaria vaccine candidate. Immunol Lett. 2004;92(3):259-68.
30. Gholizadeh Z, Tavakkol-Afshari J, Nikpoor AR, Jalali SA, Jaafari MR. Enhanced immune response induced by P5 HER2/neu-derived peptide-pulsed dendritic cells as a preventive cancer vaccine. J Cell Mol Med. 2018;22(1):558-67.
31. Makvandi M, Taherkhani R, Azizolahi B. Cloning and Expression of a Secretory form of Truncated ORF2 (aa 112-607) from Hepatitis E Virus in the pVAX1 Vector. Jundishapur J Microbiol. 2017;10(11):e13543.

32. Wang G, Pan L, Zhang Y. Approaches to improved targeting of DNA vaccines. Hum Vaccin. 2011;7(12):1271-81.
33. Tsen S-WD, Paik AH, Hung C-F, Wu TC. Enhancing DNA vaccine potency by modifying the properties of antigen-presenting cells. Expert Rev Vaccines. 2007;6(2):227-39.
34. Weinberger EE, Isakovic A, Scheiblhofer S, Ramsauer C, Reiter K, Hauser-Kronberger C, et al. The influence of antigen targeting to sub-cellular compartments on the anti-allergic potential of a DNA vaccine. Vaccine. 2013;31(51):6113-21.
35. Midha S, Bhatnagar R. Anthrax protective antigen administered by DNA vaccination to distinct subcellular locations potentiates humoral and cellular immune responses. Eur J Immunol. 2009;39(1):159-77.
36. Ashok MS, Rangarajan PN. Protective efficacy of a plasmid DNA encoding Japanese encephalitis virus envelope protein fused to tissue plasminogen activator signal sequences: studies in a murine intracerebral virus challenge model. Vaccine. 2002;20(11-12):1563-70.
37. Liang R, Van den Hurk JV, Zheng C, Yu H, Pontarollo RA, Babiuk LA. Immunization with plasmid DNA encoding a truncated, secreted form of the bovine viral diarrhea virus E2 protein elicits strong humoral and cellular immune responses. Vaccine. 2005;23(45):5252-62.
38. Luo M, Tao P, Li J, Zhou S, Guo D, Pan Z. Immunization with plasmid DNA encoding influenza A virus nucleoprotein fused to a tissue plasminogen activator signal sequence elicits strong immune responses and protection against H5N1 challenge in mice. J Virol Methods. 2008;154(1):121-7.
39. Kim SJ, Suh D, Park SE, Park J-S, Byun H-M, Lee C, et al. Enhanced immunogenicity of DNA fusion vaccine encoding secreted hepatitis B surface antigen and chemokine RANTES. Virology. 2003;314(1):84-91.
40. Li J, Liang Q, Song W, Marchisio MA. Nucleotides upstream of the Kozak sequence strongly influence gene expression in the yeast S. cerevisiae. J Biol Eng. 2017;11(1):25-9.
41. Hasanzadeh S, Habibi M, Shokrgozar MA, Ahangari Cohan R, Ahmadi K, Asadi Karam MR, et al. In silico analysis and in vivo assessment of a novel epitope-based vaccine candidate against uropathogenic Escherichia coli. Sci Rep. 2020;10(1):16258-62.
42. Daifalla NS, Bayih AG, Gedamu L. Differential Immune Response against Recombinant Leishmania donovani Peroxidoxin 1 and Peroxidoxin 2 Proteins in BALB/c Mice. J Immunol Res. 2015;2015:348401.
43. Kim D, Hoory T, Wu T-C, Hung C-F. Enhancing DNA vaccine potency by combining a strategy to prolong dendritic cell life and intracellular targeting strategies with a strategy to boost CD4+ T cells. Hum Gene Ther. 2007;18(11):1129-40.
44. Yang B, Yang A, Peng S, Pang X, Roden RBS, Wu TC, et al. Co-administration with DNA encoding papillomavirus capsid proteins enhances the antitumor effects generated by therapeutic HPV DNA vaccination. Cell Biosci. 2015;5(1):35-8.
45. Rose RC, Lane C, Wilson S, Suzich JA, Rybicki E, Williamson A-L. Oral vaccination of mice with human papillomavirus virus-like particles induces systemic virus-neutralizing antibodies. Vaccine. 1999;17(17):2129-35.
46. Gerber S, Lane C, Brown DM, Lord E, DiLorenzo M, Clements J, et al. Human papillomavirus virus-like particles are efficient oral immunogens when coadministered with Escherichia coli heat-labile enterotoxin mutant R192G or CpG DNA. J Virol. 2001;75(10):4752-60.
47. Kim S, Lee C, Lee S, Kim I, Park J, Sasagawa T, et al. Enhanced immunogenicity of human papillomavirus 16 L1 genetic vaccines fused to an ER-targeting secretory signal peptide and RANTES. Gene Ther. 2003;10(15):1268-73.
48. Hung C-F, Tsai Y-C, He L, Wu TC. DNA Vaccines Encoding Ii-PADRE Generates Potent PADRE-specific CD4+ T-Cell Immune Responses and Enhances Vaccine Potency. Mol Ther. 2007;15(6):1211-9.
49. Ghaffari-Nazari H, Tavakkol-Afshari J, Jaafari MR, Tahaghoghi-Hajghorbani S, Masoumi E, Jalali SA. Improving Multi-Epitope Long Peptide Vaccine Potency by Using a Strategy that Enhances CD4+ T Help in BALB/c Mice. PLoS One. 2015;10(11):e0142563.
50. Hung C-F, Tsai Y-C, He L, Wu TC. DNA vaccines encoding Ii-PADRE generates potent PADRE-specific CD4+ T-cell immune responses and enhances vaccine potency. Mol Ther. 2007;15(6):1211-9.
51. Wu A, Zeng Q, Kang TH, Peng S, Roosinovich E, Pai SI, et al. Innovative DNA vaccine for human papillomavirus (HPV)-associated head and neck cancer. Gene Ther. 2011;18(3):304-12.
52. Kim D, Hoory T, Monie A, Ting JP, Hung CF, Wu TC. Enhancement of DNA vaccine potency through coadministration of CIITA DNA with DNA vaccines via gene gun. J Immunol. 2008;180(10):7019-27.

53. Dan X, Han L, Riaz H, Luo X, Liu X, Chong Z, et al. Construction and evaluation of the novel DNA vaccine harboring the inhibin α (1-32) and the RF-amide related peptide-3 genes for improving fertility in mice. Exp Anim. 2016;65(1):17-25.
54. Delogu G, Li A, Repique C, Collins F, Morris SL. DNA vaccine combinations expressing either tissue plasminogen activator signal sequence fusion proteins or ubiquitin-conjugated antigens induce sustained protective immunity in a mouse model of pulmonary tuberculosis. Infect Immun. 2002;70(1):292-302.
55. Luo M, Tao P, Li J, Zhou S, Guo D, Pan Z. Immunization with plasmid DNA encoding influenza A virus nucleoprotein fused to a tissue plasminogen activator signal sequence elicits strong immune responses and protection against H5N1 challenge in mice. J Virol Methods. 2008;154(1-2):121-7.
56. Hanke T, Ondondo B, Abdul-Jawad S, Roshorm Y, Bridgeman A. Vector delivery-dependant effect of human tissue plasminogen activator signal peptide on vaccine induction of T cells. Journal of HIV and AIDS. 2016.
57. Haddad D, Liljeqvist S, Ståhl S, Andersson I, Perlmann P, Berzins K, et al. Comparative study of DNA-based immunization vectors: effect of secretion signals on the antibody responses in mice. FEMS Immunol Med Microbiol 1997;18(3):193-202.