

Antitumor Response to a Codon-Optimized HPV-16 E7/HSP70 Fusion Antigen DNA Vaccine

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ABSTRACT

Background: Vaccines based on virus-like particles are effective against Human Papilloma Virus (HPV) infection; however, they have not shown a therapeutic effect against HPV-associated diseases. New immunotherapy strategies based on immune responses against tumor antigens can positively affect the clearance of HPV-associated lesions. **Objective:** To generate two therapeutic fusion DNA vaccines (optimizedE7/mouseHSP70 and wildE7/mouseHSP70) to induce antitumor specific responses in mice models. **Methods:** Mice were immunized with recombinant DNA vaccines. The splenocytes of immunized mice were collected and lactate dehydrogenase and IFN- γ productions were measured after three injections in order to evaluate cytotoxic T lymphocytes (CTLs) activity. MTT assay was carried out for lymphocyte stimulation. **Results:** The fusion DNA vaccines, specifically uE7-HSP70, elicited varying levels of IFN- γ and CTLs responses compared to the control group ($P < 0.05$). Furthermore, antitumor response and tumor size reduction in fusion DNA vaccines groups were significantly higher than in the negative control group ($P < 0.05$). **Conclusion:** It is concluded that our fusion DNA vaccines considerably enhanced specific cellular responses against HPV tumor model. In addition, optimized E7 showed a notable immunogenicity and inhibitory effect on the reduction of tumor size.

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Keywords: Cervical Cancer, DNA Vaccine, E7 Protein, Heat Shock Protein 70, Papilloma Virus

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INTRODUCTION

Cervical carcinoma is the third most common malignancy among women around the world and the second cause of cancer death in women in developing countries (1), where, approximately, 70% of cervical cancers occur (2,3). Numerous studies have shown that the development of this cancer is closely associated with infection by high-risk types of Human papilloma virus (HPV), particularly HPV-16 and 18 (4). HPV prophylactic vaccines (Gardasil and Cervarix) are very efficient and capable of preventing infections caused by viruses, because they can elicit strong systemic immune responses, including neutralizing antibodies. These vaccines, on the other hand, are not able to eliminate pre-existing infection and HPV-related lesions (5). HPV-encoded oncoproteins, particularly E7, are highly expressed and essential for viral transformation in cancer cells; accordingly, in HPV therapeutic vaccines, viral oncoproteins are ideal target antigens for the induction of specific antitumor responses in the development of vaccines and immunotherapy strategies against HPV-associated neoplasms (6). An effective therapeutic vaccination against HPV-related cancer mostly depends on the induction of specific cellular immune responses in infected humans. Codon optimization is a commonly used strategy to enhance cellular immune responses by the DNA vaccine and is related to the modification of antigenic nucleic acid sequences by replacing codons that are rarely recognized by cellular protein synthesis machinery with codons that are more commonly recognized (7,8). Recently, the effect of optimized codons on the immunogenicity of polynucleotide vaccines for viral infection has been the subject of certain studies. Papilloma virus capsid protein expression is dependent on the match between codon usage and tRNA availability in target cells, and, when delivered by the gene gun, codon modified HPV16 L1 and L2 polynucleotide vaccines are more immunogenic than unchanged vaccines (7,8). Nonetheless, certain other studies have demonstrated that mice immunized with codon-optimized HPV-16 E6 DNA enhanced antigen-specific CD8⁺T cell immune responses compared to mice immunized with wild-type E6 DNA (8). Moreover, the fusion of optimized HPV-16 E7 with heat shock protein-70 (HSP70), as a powerful adjuvant, may be a strategy to ameliorate cellular immune responses to a DNA vaccine. The use of HSP70 in the background of DNA vaccines is yet another promising approach for enhancing cell-mediated immune responses and DNA vaccine development (9). Immunological functions of HSP70 can be divided into three major areas: i) chaperoning and protein folding properties where HSP70 can bind to tumor antigen peptides and enter the antigen processing and presentation pathway (9,10). ii) Cross-presentation function; through binding to endocytic receptors on APC, HSP70 can participate in the MHC-I pathway and facilitate cross-presentation of the associated antigens and augment antigen-specific CTLs responses (9,10). iii) Pro-inflammatory activities where HSP-70 can induce the activation and maturation of dendritic cells (DCs) by binding to the receptors of CD40, TLR-2, TLR-4 on the DC. Subsequent activities are characterized by the up-regulation of MHC-II, CD86 and CD83 expression, and secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6 and IFN- γ (11-13). Here, we used the optimized E7 oncogene to significantly improve the weak immunogenicity of the HPV tumor antigen E7. As an alternative approach, the injection of recombinant DNA vaccine that co-express optimized E7 and HSP-70 can improve the immunogenicity of an HPV16 E7 DNA vaccine over those induced by E7 vaccine alone, rendering it an efficacious therapeutic vaccine against HPV.

MATERIALS AND METHODS

Construction of recombinant DNA vaccine. To promote HPV16 E7 expression, codons of this gene were assessed and optimized using computer-based modeling. The final nucleic acid sequences were designed and synthesized by GeneScript Co. (Piscataway, USA) and cloned into the plasmid PUC18\19. The fragment bearing the full region of the optimized E7/wild E7 genes from PUC18\19 was purified and ligated into plasmid pcDNA3.1+ as described below. Briefly, KpnI and BamHI sequences and three overhanging nucleotides are at the beginning of forward and reverse primers, respectively. The forward primer contains the kozak consensus sequence and start codon. A stop codon mutation was inserted in the 3' end of the uE7 and wE7 genes so that HSP70 peptide was translated into the same frame of the start codon of the corresponding genes. The final schema of fusion gene is illustrated in Figure 1. The amplified wild-type and codon-optimized E7 genes were cloned into the pTZ57R/T cloning vector (Thermo scientific, USA). Wild type and codon-optimized E7 genes were then subcloned into pcDNA3.1+ mammalian expression vector (Invitrogen, Carlsbad, CA) downstream of the cytomegalovirus promoter. Colonies are screened by an antibiotic resistance marker, colony-PCR and restriction enzyme analysis. So as to generate E7-HSP70 chimera, two primers containing the linkers and BamHI and XhoI restriction enzyme site were designed for the amplification of HSP70 gene. The PCR product of modified HSP70 was purified from the agarose gel by a purification kit (Bioneer, Korea), and further employed for the cloning process. The HSP70 DNA was subcloned to the 3' end of pcDNA3.1+ containing uE7 and wE7. All recombinant clones were confirmed using colony-PCR, restriction enzyme digestion (Figure 2) and sequencing. The oligonucleotide primers used in this study (Metabion, Germany) are shown in Table 1. Kozak sequence with the start codon (ACCATG) was added at the 5'-end of the forward primers sequence. The termination codons were eliminated at the 3'-end of the reverse primers sequence. At both sides of forward and reverse primers, two restricted enzyme sites (KpnI and BamHI) were designed. A linker sequence was added into the forward primer of HSP70 and also an XhoI site was added after stop codon (TAA) of reverse primer (nucleotides are underlined).

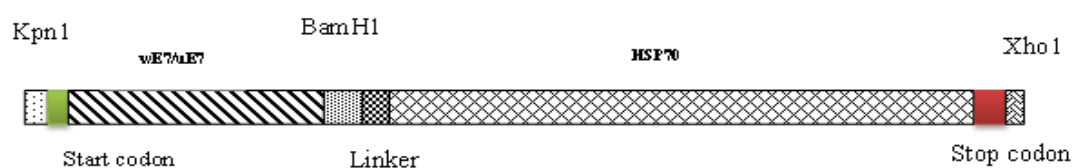


Figure 1. The diagram of the recombinant fusion gene. To generate the fusion constructs, the termination codon of HPV-16 uE7/wE7 mutations were deleted and linked with HSP70 by a spacer of Glycine, Glycine, Glycine. The kozak sequence with the start codon (ACCATG) was added at the 5'-end of the DNA sequence. At both sides of the sequence, two restricted enzyme sites (KpnI and XhoI) were predicted. Also, a stop codon (TAA) was added at the 3'-end of the target sequences.

Table 1. Oligonucleotide primers used in this study (Metabion, Germany).

Gene	Primer (5'→3')	Size
<i>uE7</i>	F: TCCGGT <u>ACCAT</u> GGGCGACACC R: TCCGGT <u>ACCAT</u> GGGCGACACC	330 bp
<i>wE7</i>	F: TTGCCGGT <u>ACCAT</u> GGATGGAG R: AGCTC <u>GGATC</u> CTGGTTTCTGAG	330 bp
<i>hsp70</i>	F: <u>AGGATCCGGC</u> ATGGCCAAAGC R: GA <u>ACTCGAG</u> CTAATCTACCTCCTCAATG	1800bp

***In vitro* evaluation of transient expression in Cos 7.** In order to monitor the expression of uE7/HSP70 and wE7/HSP70 recombinant proteins, 106 Cos7 cells were seeded into a 6-well microplate and incubated overnight in an antibiotic free complete medium. The cells were used for transfection at 70% confluence. The transfection was performed via pcDNA3.1+/uE7-HSP70 and pcDNA3.1+/wE7-HSP70 or pcDNA3.1+ using Lipofectamine 2000 (Invitrogen, Germany) according to the manufacturer's recommendations. After 48 h, the cells were scrapped and the total lysates were prepared by freezing and thawing (four times) followed by sonication (60 HZ, 0.5 Amplitude for 60 sec). Phenyl Methane Sulfony Fluoride (PMSF, 1 mM) was added to the cell lysates to inactivate the cellular proteinase. The lysates were centrifuged (800 g), and the protein was quantitated by Bradford assay. The cell lysates were separated on 12% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose paper for 18-24 h at 25 mV and 40C. For one hour, the paper was incubated with mouse anti-HSP70 antibody (Abcam, UK) and three times washed by 15 ml of phosphate buffered saline containing 5% dried milk for 10 min. To detect the antigen-antibody complex, the membrane was washed and then incubated with horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin G antibody (1:1000) for 1 h at room temperature. The membranes were washed 3×20 min with TBS-T, and the bonds were detected through the use of diaminobenzidine (DAB) substrate (Biogen, Germany). The expression of both the uE7 and wE7 proteins has been previously corroborated (14).

Mice and cell lines. TC-1 cell line was purchased from the Pasteur Institute, Tehran, Iran. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 cell-culture medium (Gibco Invitrogen; Paisley, Scotland, UK), supplemented with 10% fetal calf serum (FCS) (Gibco BRL), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, and 5% CO₂ at 37°C. Sixty-three female C57BL/6 mice (3-4 week old) were purchased from the Pasteur Institute (Tehran, Iran). The mice were housed for one week prior to the experiment, given free access to food and water, and maintained in a good standard condition. All experiments were carried out on the basis of the guidelines for the care and use of laboratory animals by the Ethical Commission of the Tarbiat Modares University.

Mice immunization. All mice were injected subcutaneously with 100 µg DNA three times over 7-day intervals. To evaluate the uE7-HSP70 recombinant protein with regards to its induction of cytotoxic T-cells response, several groups of mice were

immunized subcutaneously with phosphate buffered saline (PBS) (negative control), pcDNA3.1+ (negative plasmid control), pcDNA3.1+ wE7, pcDNA3.1+ uE7, pcDNA3.1+ wE7/HSP70, pcDNA3.1+ uE7/HSP70, pcDNA3.1+ wE7+HSP70, pcDNA3.1+ uE7+HSP70 and pcDNA3.1+HSP70. Finally, four mice in each group were randomly sacrificed two weeks following the last immunization.

Lymphocyte proliferation assay. Single suspension of the spleen mononuclear cells (MNCs) was harvested from immunized and negative control groups and used for lymphocyte proliferation assay (LPA). In a nutshell, the suspension of cells was cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine, 1% HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)], 0.1 mM nonessential amino acid, and 0.1% penicillin/streptomycin and further incubated in the presence of 5 µg/ml TC-1 cell lysates per well. A total of 100 µl of medium alone and 5 µg of phytohemagglutinin (PHA) (Sigma Chemical Co, St Louis, MO, USA) were added to the well as negative and positive control, respectively. All plates were incubated at 37°C for 72 h in a humidified atmosphere containing 5% CO₂. Furthermore, 100 µl aliquot of supernatant was removed and 20 µl of MTT (3-(4, 5-dimethyl tetrazolyl-2) 2, 5 diphenyl) tetrazolium bromide (Sigma Chemical Co, St Louis, MO, USA) with a concentration of 5 µg/ml was added per well and incubated for an additional 5 h at 37°C in the presence of 5% CO₂. Next, 100 µl dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals produced by proliferating cells. After incubating the plates at 37°C for 15 min, stimulation index (SI) was specified at 540 nm absorbance as follows: The OD of stimulated cells (Cs) was subtracted from the OD of unstimulated cells (Cu) by the OD measurement of unstimulated cells. All tests were performed in triplicate for each mouse (15).

Cytotoxic T-lymphocyte assays. The CTL activity of the spleen MNCs (as effector cells) belonging to the various treated mice was assayed immediately using the lactate dehydrogenase (LDH) release assay, according to manufacturer's instructions (Takara Biotechnology Co., Ltd., Dalian, Japan). Different concentrations of effector cells and E7-loaded EL4 cells (100:1, 50:1, and 25:1) were cocultured for 4 h in phenol red-free RPMI 1640 containing 3% FCS. Following centrifugation at 250 g for 10 min, the supernatants (50 µl/well) were transferred to the 96-well round-bottom plates, and the lyses of target cells were determined through measuring LDH release. Several controls were used for cytotoxicity assay. "High Control" (HC) was the total LDH released from the target cells, and all EL4 cells were lysed by a medium containing 1% Triton X-100. "Low Control" (LC) was the natural release of LDH from the target cells, which was induced by adding EL4 cells only to the assay medium. The "T cell control" (efC) was used to evaluate the natural release of LDH from T cells induced by adding different ratios of T cells only to the assay medium. All sample assays, including the controls, were performed in triplicate. The percentage of the specific release of LDH (%) was calculated via the following formula (16):

$$= 100 \times (A_{ef} - A_{tar} - A_{efC}) / (A_{hC} - A_{lC})$$

Where index A is A₄₉₂ value for effector (ef), target (tar) cells and control cells (see above).

Cytokine ELISA assay. Splenic lymphocytes were incubated as previously described for the proliferation assay. After 72 h, supernatants were collected and analyzed in order to detect the presence of different cytokines. The levels of IFN-γ and IL-4 production were determined by using commercially available sandwich-based ELISA kits (R&D

Systems, Minneapolis, Minn., USA). The lower limit of detection for the cytokine was 32 pg/ml and all tests were performed in triplicate for each mouse.

Tumor monitoring: The TC-1 cells, derived from the primary lung epithelial cells of C57BL/6 mice and immortalized with the amphotropic retrovirus vector containing E6 and E7 genes, were used as tumor cell model. For this purpose, C57BL/6 mice were challenged by subcutaneous injection in the left flank with a suspension of 100 μ l PBS containing 10⁶ TC-1 cells/mouse. Following one week, the mice were subjected to several DNA vaccines. The neoplastic masses were measured with calipers every other day and the tumor volume was estimated according to Carlsson's formula (17). The remaining mice were examined over 60 days to check the appearance and size of the tumors. The average tumor size in millimeter is reported as the average of all measured dimensions. The smallest diameter (a) and the biggest diameter (b) were measured and tumor volume was calculated using the $V = (ab^2)/2$ formula.

Statistical analysis. The GraphPad Prism 6.01 software (La Jolla, CA, USA) was used for statistical analysis. The significance of statistical comparisons was calculated using one way ANOVA. The p-values less than 0.05 were considered significant and values were expressed as means \pm SD.

RESULTS

wE7-HSP70 and uE7-HSP70 fusion constructs.

Chimeric wE7-HSP70 and uE7-HSP70 were constructed by linking the full length wE7 and uE7 to HSP70 containing a stop codon for proper protein production. The presence of the desired plasmids in bacteria was confirmed by colony-PCR using F1/R3 and F2/R3 primers. The colonies containing the desired plasmids were determined by the production of a DNA band around 2100 bp. Restriction enzyme digestion and sequencing analysis confirmed both wE7-HSP70 and uE7-HSP70 recombinants (Figure 2).

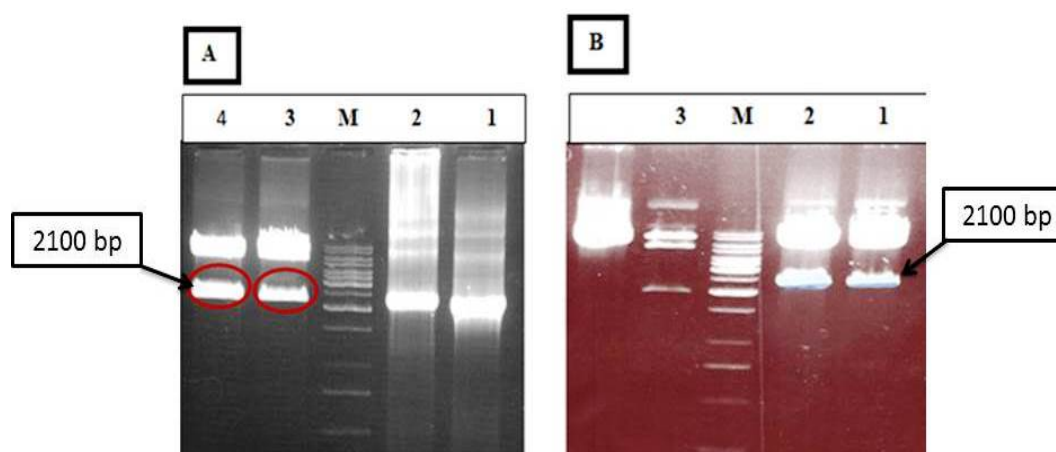


Figure 2. Colony PCR and double digestion of resultant constructed plasmids. A) The recombinant plasmids pcDNA3.1+ uE7/Hsp70 and wE7/Hsp70 were identified by colony PCR (Lane 1,2) and enzyme digestion (Kpn1 and Xho1) (Lane 3,4). Lane M is the 1kb DNA ladder. **B)** The results pertaining to the enzyme digestion analysis of recombinant plasmids (pcDNA3.1+ uE7/Hsp70 and wE7/Hsp70) (Lane 1,2) were compared with the enzyme digestion of pcDNA3.1+ HSP70 (Lane 3). Lane M is the 1kb DNA ladder.

Expression of wE7/HSP70 and uE7/HSP70 fusion proteins.

Western blot analysis of cell lysates indicated proteins band with a size of approximately 82KDa representing both wE7/HSP70 and uE7/HSP70 DNA recombinants (Figure 3). These results confirmed the expression of the proteins as they migrated based on their corresponding size.

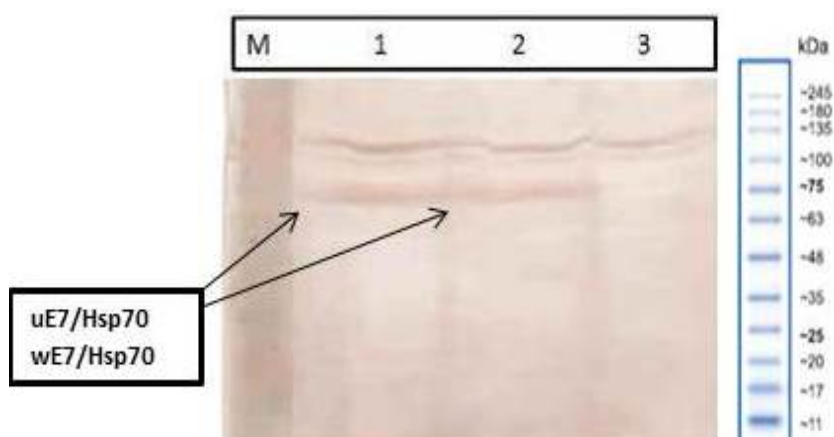


Figure 3. Detection of fusion proteins in Cos7 cells via Western blot analysis. Western blot analysis by mouse anti-HSP70 antibody showed negative results in pcDNA3.1+ transfected cells (Lane 3) and two fusion protein bands with a size of approximately 82KDa in the transfected cells with pcDNA3.1+ uE7/HSP70 (Lane 1) and pcDNA3.1+ wE7/HSP70 E7 (Lane 2).

LDH cytolytic responses.

The CTL response in immunized mice was examined in this study by the LDH release assay in 96-well plates. Results show significantly enhanced CTL responses against uE7/HSP70 and wE7/HSP70 fusion genes. As shown in Figure 4, at an E/T ratio of 50:1, lymphocytes from the mice vaccinated with uE7/HSP70 (67 ± 2.6) had a significantly higher specific cytolytic activity compared with those vaccinated with PBS ($12.1\% \pm 0.70\%$), pcDNA3.1+ (negative plasmid control $19.1\% \pm 0.99\%$), pcDNA3.1+ wE7 ($37.4\% \pm 2.09\%$), or pcDNA3.1+ uE7 ($41.3\% \pm 2.2\%$) ($P < 0.05$). However, no statistically significant difference was found between uE7/HSP70 and wE7/HSP70 groups as far as in cytolytic activity is concerned. At E/T ratios of 25:1 or 100:1, there was no noticeable difference between the groups.

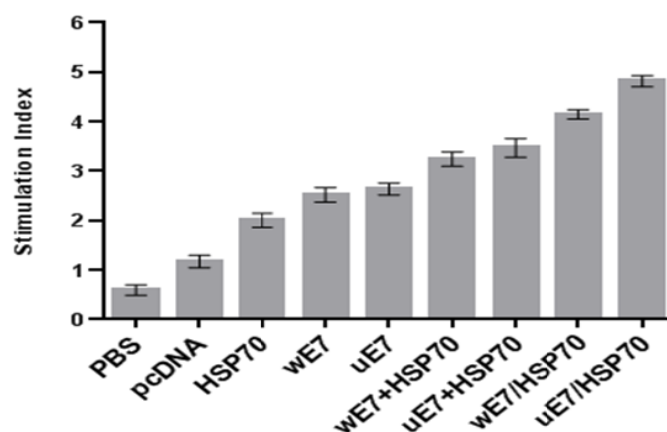


Figure 4. Quantitative measurement of LDH release in immunized mice. Data were collected from LDH results at E/T ratios of 50:1 and expressed as percent cytotoxicity \pm SD. The data demonstrated here are from three independent experiments in triplicate.

T cell proliferation response.

The lymphocyte proliferation in response to the specific antigen re-stimulation was measured using MTT assay; stimulation index (SI) was subsequently calculated with the results pointing to the fact that the SI in uE7/HSP70 and wE7/HSP70 fusion genes vaccinated groups was higher than that in PBS, pcDNA3.1+ (negative plasmid control), pcDNA3.1+ wE7 or pcDNA3.1+ uE7 ($p < 0.05$) groups (Figure 5). No statistically recognizable difference was observed between the uE7/HSP70 and wE7/HSP70 groups regarding SI.

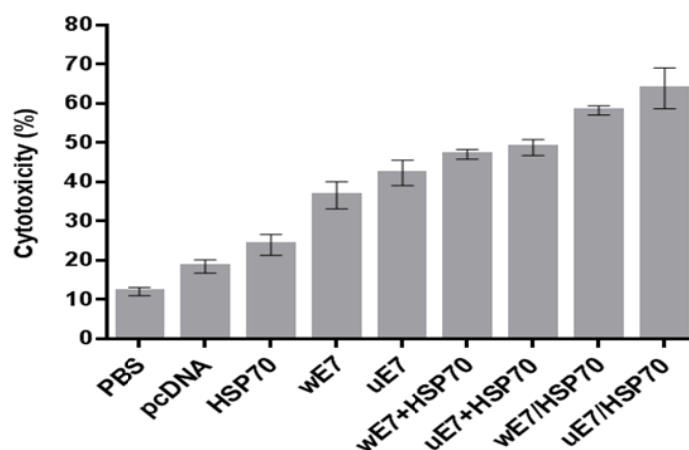


Figure 5. Splenocyte proliferation levels following *in vitro* re-stimulation with specific antigen. The results show that the stimulation index values of mice vaccinated with uE7/HSP70 and wE7/HSP70 increase more significantly than other groups ($p < 0.05$).

Secretion levels of cytokines.

The level of IL-4 and IFN- γ were assessed by ELISA assay in order to determine whether or not vaccination with DNA vaccines is capable of upregulating cytokine secretion and increasing specific immune responses in the vaccinated mice. Results show that IFN- γ level significantly increased in the uE7/HSP70 and wE7/HSP70 vaccinated mice (470.33 ± 66 and 436.4 ± 55 , respectively) compared with other groups ($P < 0.01$). IL-4 assay did not induce any statistically significant change among the groups (Figure 6).

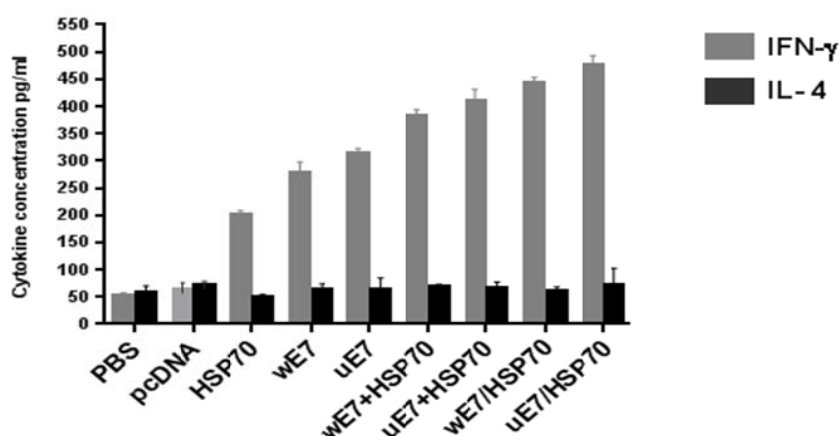


Figure 6. Determination of the production of Th1 and Th2 cytokines by the splenocytes of immunized mice. The statistical difference levels between test groups were determined using ANOVA where each sample was examined in triplicate ($p < 0.05$).

In vivo tumor challenge assays.

As shown in Figure 7, mice treated with uE7/HSP70 and wE7/HSP70 fusion vaccines underwent significant decrease in tumor volumes, in comparison with mice treated with PBS, pcDNA3.1+, pcDNA3.1+ wE7, or pcDNA3.1/uE7 ($P < 0.05$). The results indicate that vaccination with uE7/HSP70 and wE7/HSP70 induced a more efficiently therapeutic antitumor effect than vaccination in the control groups.

DISCUSSION

Nucleic acid vaccines are highly acceptable for generating antigen-specific immunity owing to their stability and simplicity of delivery (1). Numerous studies have tested different strategies with the objective of enhancing and directing the potency of DNA vaccines (3,18-21). These strategies include selecting the injection site, improving the plasmid delivery system, utilizing suitable expression vectors, prime-boost strategy, enhancing formulation with proper adjuvants, and codon optimization strategy (3,18-21).

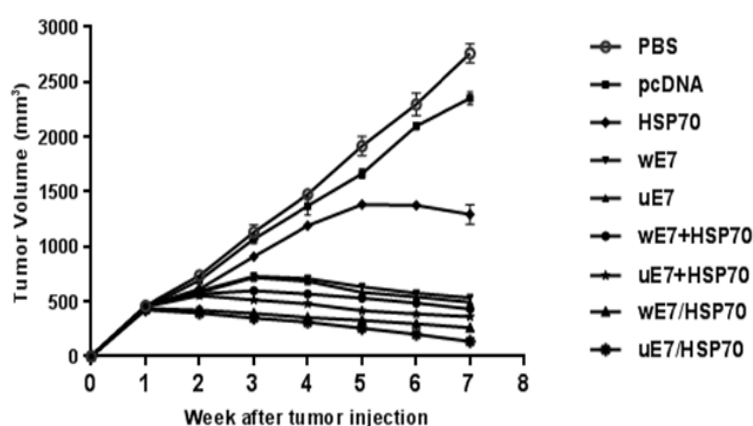


Figure 7. Assessment of the effect of therapeutic vaccine on tumor size between the 5th and 7th weeks following tumor inoculation. Results show that the differences in uE7/HSP70 and wE7/HSP70 vaccinated groups were statistically significant ($p < 0.05$). The tumor growing curve of the pcDNA3.1+ uE7/HSP70 group was the lowest among the mice in the nine groups. Line and scatter plot graphs depicting the tumor volume (in mm^3) are presented. The data presented are a representation of two independent experiments.

The low *in vivo* expression of E7 genes is a common challenge when using this gene in the context of DNA vaccines, reducing protein immunogenicity, and rendering it difficult to identify protein band through protein-based methods (7,8). In the study of Liu *et al.* Cos-1 cells transfected with optimized E7 expressed higher levels of E7 protein than similar cells transfected with wild E7 alone. In addition, they demonstrated that the optimized E7 induced a significantly stronger E7-specific cytotoxic T-lymphocyte response than wild E7 and a 100% tumor protection in C57BL/6 mice model (7). Similar to Liu, we provided evidence as to the fact that codon optimization strategy and HSP70 conduce to increasing E7 gene expression and enhancing anti-tumor immune responses in animal models. We found an increased level of IFN- γ in mice vaccinated with uE7/HSP70 or wE7/HSP70. In contrast, mice immunized with wE7 and uE7, alone or in combination with HSP70 had a reasonable level of IFN- γ secretion compared with the control groups as shown in figures. Considering the fact that IFN- γ significantly affects tumor growth and apoptosis induction by stimulating the CTL cytotoxic activity (22), we observed a significant decrease in tumor size in mice vaccinated with our fusion DNA vaccines. These results are in line with that of Oosterhuis *et al.* in which they generated E7-specific CD8 T cells through the use of a fusion HPV vaccine containing tetanus toxin fragment C to produce IFN- γ , increase cytolytic activity and eradicate tumor (23). In another study, Zong and colleagues illustrated that vaccination with the E7/HuHSP70 DNA vaccine induced a stronger E7-specific CD8 T cell immune response than E7 vaccine and resulted in a more significant therapeutic effect against E7-expressing tumor cells (24). In general, results of such ilk are indicative of the fact that E7-specific CMI responses, including IFN- γ production, increased proliferation of CD8⁺ and their cytotoxicity effect play a critical role in reducing HPV-induced tumors.

In conclusion, our DNA vaccines can be employed with other stimulating agents including, GM-SCF, listeriolysin O and HSPs (GP96) to induce strongly cellular

immune responses. More studies are required to fully understanding of the effect of such therapies on specific tumor models. The evaluation of anti-HPV16 neutralizing antibodies plays a key role in the prevention of HPV16-associated cancer and is to be minutely investigated.

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