NSP4 as Adjuvant for Immunogenicity and Design of Effective Therapeutic HPV16 E6/E7/L1 DNA Vaccine in Tumoric and Healthy C57BL/6 Mice

Sahar Sadr-Momtaz

Golestan University of Medical Sciences

Maryam Aftabi

Golestan University of Medical Sciences

Emad Behboudi

Golestan University of Medical Sciences

Malihe Naderi

Shahid Beheshti University

Anahita Hashemzadeh-Omran

Golestan University of Medical Sciences

Abdolvahab Moradi (✉ abmoradi@gmail.com)

Golestan University of Medical Sciences

Research Article

Keywords: Vaccine, HPV, E6/E7/L1, Adjuvant, NSP4

Posted Date: December 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1176705/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Introduction: In humans, approximately 5% of all cancers are attributable to HPV infection. Prophylactic vaccines can inhibit viral migration and persistence. However, requirement to develop therapeutic treatments prevails. To achieve this goal, we designed a therapeutic HPV DNA vaccine encoding a construct of E6/E7/L1 and used NSP4 antigen as adjuvant to assess efficiency of this construct in generating antigen-specific antitumor immune responses.

Material and Methods: Sixty female C57BL/6 mice (6–8 weeks old) were purchased from Institute Pasteur of Iran. 30 of them became cancerous, but 30 of them were healthy control. To amplify E6/E7/L1-pcDNA3, NSP4-pcDNA3, expression vector of DH5α and TC-1 cell line were used to generate a tumor. Mice were immunized with HPV DNA vaccine. Cell proliferation was assessed by MTT assay. Finally, we assessed cytokine responses (IL-2, IL-4, INF-γ), in the serum of mice spleen cells.

Result: Mice receiving the NSP4/E6-E7-L1 vaccine had the highest stimulatory index compared to other groups but it was not significant. Interleukin 4/12 and INF-γ production were significantly higher in E6-E7-L1 / NSP4 group and E6-E7-L1 group compared to other groups ($P < 0.05$). Among different groups, E6/E7/L1 + NSP4 group was able to slow down the tumor growth process, but it was not significant ($p > 0.05$). Among the cytokines mentioned, IFN-γ and IL-12 are among the cytokines that stimulate the Th1 pathway and IL-4 cytokine stimulates the Th2 pathway and B lymphocytes.

Conclusion: Our data suggest that present vaccine can stimulate innate and acquired immunity response, and can be a therapeutic vaccine in the tumoric mice.

Introduction

Human papillomavirus (HPV) is a major etiological pathogen in cancers, such as cervical, vulvar, vaginal, anal, penile, and head and neck tumors. Cervical cancer immunotherapy must target high-risk HPV16, 18 that cause 50% and 20% of cervical cancers, respectively [1, 2]. Prophylactic vaccines can inhibit HPV migration and persistence, but HPV induced cancer is still the reason of more than 200000 deaths in year in populations. Therefore, the need to develop therapeutic treatments prevails [3, 4].

The best therapeutic HPV vaccine should be capable to both eliminating virions and transformed cells expressing viral proteins. The viral oncoproteins (E6/E7) are appropriate antigen targets for immunotherapy, because they are key factors in cell transformation and they are constantly expressed by HPV-related neoplastic epithelial and tumor cells (4). The cellular immune response is responsible for removing HPV transformed cells. Thus, optimal therapeutic vaccines should be designed in a way to raise adaptive response. Several experimental therapeutic vaccines for HPV related oncogenesis have been introduced in trials but majority of them had only partial progresses. These models were adjuvanted proteins, DNA vaccines, live vaccines, or dendritic cell-related vaccines [3].

However prophylactic HPV vaccines are designing to avoid of HPV infection spreading through triggering a considerable antiviral antibody response toward viral L proteins. Today there is two approved preventive HPV vaccines which are available in market (Cervarix and Gardasil) and their efficacy to decreasing the incidence of high-level cervical abnormalities have been confirmed [5]. But this is noticeable that, due to integration of HPV genome in the host genome, the L1 and L2 proteins cannot be expressed in transformed cells in some stages of disease and this fact makes the current vaccines useless in that phase of infection [2, 6].

In the other hand, DNA vaccines are capable to trigger the immune response by multiple mechanisms in the same time, instantly, through designing several target antigens or combining immune-modulatory factors [7]. Furthermore, using of these vaccines is so beneficial because they are resistant to temperature changings and also cost-effective, and these properties make them a preferred vaccine choice in the worldwide. Although, DNA vaccines have shown to be weak immunogenic in clinical trials, researchers established a DNA vaccine strategy that uses codon-optimized constructs in combination to target both humoral and cellular immunity [8, 9].

Using an adjuvant is one of the approaches to increase the immunogenicity of these vaccines. Recently researchers showed that Rotavirus nonstructural protein 4 (NSP4) has immune response stimulation functions [10]. Due to the NSP4 adjuvant properties for increasing the efficiency of vaccine, we used NSP4 coding plasmid together with E6/E7/L1 coding plasmid. Moreover, codon-optimization, which was primarily applied to enhance protein expression, should also limit the possibility of recombination of the vaccine sequences with wild-type HPV viruses [8]. In this study, we designed a therapeutic HPV DNA vaccine encoding a construct of E6/E7/L1 and used NSP4 antigen as adjuvant in C57BL/6 mice. Furthermore, we assessed efficiency of this construct in generating antigen-specific antitumor immune responses to protect mice from tumor challenge and to treat preexisting tumor in mice. Finally, we determined the immunity type that is responsible for the observed antitumor responses in vaccinated, tumor challenged mice.

Material & Methods

- Bacterial strain and cell line

The DH5 α strain of *Escherichia coli* competent cells was used as a bacterial host for transformation in presence of calcium chloride and heat shock treatment, based on the method of Cohen *et al.* [11]. Briefly, *E. coli* strain DH5 α was cultured at 37 °C in Luria Bertani (LB) medium, supplemented with 50 μ g/mL ampicillin. TC-1 cell line (primary epithelial cell of the lung of C57BL / 6 mice) was cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco BRL, Paisley, UK) supplemented with 2 mM/L L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, 5 $\times$ 10⁻⁵ M β -mercaptoethanol, and 10% fetal bovine serum (FBS) at 70% confluency 20h before transfection.

- DNA vaccine construction

We provided a pcDNA3.1 plasmid containing E6/E7/L1 gene expression cassette under human cytomegalovirus (HCMV) immediate-early promoter and NSP4-pcDNA3.1 expression vector Escherichia coli strain DH5α from Tarbiat Modares University. Purified polymerase chain reaction (PCR) products were cloned into a pcDNA3.1 cloning vector and were confirmed by sequencing. The digested gene was sub-cloned into a pcDNA3.1 eukaryotic expression vector (Invitrogen, Canada). E. coli DH5α strain bacteria were transformed with the plasmids and plated on LB plates containing 100 µg/mL ampicillin. The selected colonies were extracted using a Gene All kit according to the manufacturer's instructions. Plasmid pcDNA3.1-NSP4 was kindly provided by Dr Razavi (Golestan university of medical science). Cloning confirmation was done by restriction enzyme digestion and PCR.

- Tumor challenge

For the therapeutic experiments, C57BL/6 mice were challenged by a subcutaneous (s.c) injection of a suspension of 100 µl PBS containing 10⁶ TC-1 cells/mouse in the back side and then, were grouped into six cages. Flow diagram of experiment was as Fig.1.

- Vaccination

In this study sixty female C57BL/6 mice with Six- to eight-week-old were bought from the Tehran Institute. All animal experimental protocols were used according to WHO and Animal Ethics Committee of Golestan University of medical sciences approved protocols of appropriate mice care and the mice were acclimated for 1 week before the experiment. Mice are divided into two equal groups of 30, cancerous and healthy. Both groups were distributed in six sub-groups in the same way (Table 1).

Table 1: Mice groups in-experiment

<i>Group I</i>	Control (Tumoric Group: TC1, Healthy Group: Sham)
<i>Group II</i>	PBS
<i>Group III</i>	pcDNA3.1+ plasmid alone
<i>Group IV</i>	Plasmid pcDNA3.1+ + NSP4
<i>Group V</i>	plasmid pcDNA3.1+ E6, E7, L1
<i>Group VI</i>	plasmid pcDNA3.1+ E6, E7, L1 + NSP4 adjuvant

- Immunizations

For DNA vaccine immunizations, mice were immunized subcutaneously in the abdominal area with a total of 30 µg HPV DNA vaccine (HPV16-E6/E7/L1 and Rotavirus NSP4, secreted and ubiquitinated construct pooled 1:1 by weight). Mice were injected intraperitoneal with 200 µg of monoclonal antibodies targeting PD-L1 (clone B7-H1, BioXcell) one dose weekly for 3 weeks following the first vaccination.

- MTT

Two weeks after final immunization, spleen of each mouse was removed and lymphocyte proliferation was evaluated using the MTT method according to the manufacturer's recommendations (Sigma) by TC1 lysate. In this way, we prepared the TC1 cell by sonicating and removing the cell wall and releasing the protein.

- ELISA

One week after the last immunization, freshly isolated splenocytes of immunized mice in the different treated groups were cultured. The cell supernatants were collected and assayed for the presence of IFN- γ , IL-4 and IL-12 using commercially available sandwich-based enzyme-linked immunosorbent assay (ELISA) kits (in comparison to unstimulated controls) (Peprotech, New Jersey, US) following the manufacturer's instructions. All tests were performed in triplicate for each mouse. Among the cytokines mentioned, IFN- γ and IL-12 are among the cytokines that stimulate the Th1 pathway and IL-4 cytokine stimulates the Th2 pathway and B lymphocytes.

- Statistical Analysis

Statistical analysis was performed by Graph Pad Prism version 8.0.1. The levels of statistical significance for differences between experimental groups were determined using T-test. Survival curves were compared by a log-rank (Mantel Cox) test. Differences were considered significant (P-value <0.05).

Result

- Effectiveness of vaccination on tumor size

Seven days after injection of TC-1 cells, tumor was observed in C57BL/6 mice. Tumor size was measured at 6 different time intervals during the vaccination program. The results of measurement showed that the tumor size was lower in the vaccinated groups compared to the control groups. Interestingly, the E6/E7/L1 + NSP4 group was able to slow down the tumor growth process compared to the other groups, but it was not statistically significant ($p > 0.05$). (Fig. 2)

- Lymphocyte proliferation assay

The results revealed that the stimulation index of vaccinated groups is significantly higher than the control groups ($p < 0.05$). The mice receiving the NSP4 / E6-E7-L1 vaccine had the highest stimulatory index compared to other groups. Comparison the results of lymphocyte proliferation assay in different groups and the control group was not significant. (Figur.3).

IFN- γ , IL-4 and IL-12 secretion levels

To investigate the level of IL-4/12 and INF- γ production, spleen cells of immunized mice in different groups were cultured and also stimulated with specific antigens. Results showed that interleukin-4/12

and INF- γ production were significantly higher in E6-E7-L1 / NSP4 group and E6-E7-L1 group compared to control and other groups (Figure.4). Although, the differences between these two groups (tumoric and healthy) was not significant.

Discussion

Cancer immunotherapy using DNA vaccines have emerged as a potentially promising approach for the control of tumors. This study showed that pcDNA3.1- E6-E7-L1/NSP4 vaccine could elicit a stronger specific CD8⁺ T cell-mediated immune response than other groups. Our results showed that vaccination by pcDNA3.1-E6-E7-L1/NSP4 can decrease tumor size and could elicit potentially protective and therapeutic antitumor responses against HPV16 expressing TC-1 tumor model in mice. Also, we showed that the pcDNA3.1-E6-E7-L1/NSP4 induced antitumor immune response may be CD8⁺ T cell dependent. In the current study numerous important mechanisms contributed to the enhance immunogenicity of pcDNA3.1- E6-E7-L1/NSP4 DNA vaccine were observed.

Previous studies reported that coadministration of DNA encoding BPVL1 enhances the immunogenicity of E7 DNA vaccine by increasing CD4⁺ T cell responses that are known to assist the generation of CD8⁺ T cell response [12]. The CD8⁺ T cells served as the basis for the design of a fusion BPVL1-E7 DNA vaccine, and now we used that data for design of a E6-E7-L1/NSP4 DNA vaccine. Additionally, several studies have tested the conjugation of E7 to L1 VLPs as a method to enhance the E7 immune response [13-15]. It has been shown that intact HPV-L1 VLPs can interact with DCs to directly induce potent adaptive immune responses in the absence of adjuvants [16]. Potentially, it occurs through a TLR-MyD88 pathway-dependent manner [17]. In contrast to our results, Tahmatan *et al.* and Sajjadian *et al.* studies showed that HPV16 DNA-E7 inoculation did not increase IL-4 expression compared to the simple vector control group [18, 19]. In Rahimi *et al.* study, there was significant difference between using E7 DNA vaccine and PBS. In their study, due to the simultaneous use of both Hitchner B1 and LaSota strains with the HPV-16 E7 DNA vaccine, the expression level of IL-4 was much higher than other groups [20]. In the present study, vaccine also have the ability to stimulate humoral immunity, which can serve as a basis for preventing future infections or treating HPV infection. Many researches in the field of antitumor vaccines developed various vaccines for HPVs, but our study is one of the researches designing a preventive and therapeutic vaccine on normal and tumoric mouse models in which revealed the efficacy of a vaccine in cellular and humoral immunity.

The HPV E6 and E7 proteins have been demonstrated to be constantly expressed in cervical cancer cells[21]; thus they are rational targets for HPV-16 therapeutic vaccination in cases with cervical carcinoma. Studies revealed that endothelial cells are capable to take up the E7 viral protein added to the culture medium[22]. Moreover, the HPV-16 E6/E7⁺ cancerous cells can genetically be modulated with DNA coding for immune regulatory cytokines or adjuvants utilized for vaccination. Immunological studies had shown a key role for CD8⁺ T lymphocytes in suppression of an aggressively growing carcinoma[23].

Recent studies have revealed that HPV-16 L1 protein has stimulatory effect on cellular immunity, therefore it could be applied for manufacture of therapeutic vaccines. Cheung et al. (2004) engineered L1E7hpSCA1 vector expressing the L1 and E7 proteins with the codon optimization for mammalian cell expression. Once their vaccine was injected into the muscle, they found that this vector had strongly induced T cell cytotoxicity and also are capable to lyse the TC-1 cells expressing E7. In animal models, tumor growth was suppressed in vaccinated mice [24]. The protection toward challenge by TC-1 tumor was also revealed by vaccination with chimeric vector harboring the HPV-16 L1/L2 viral capsid proteins and the E7 oncoprotein [14]. In our study we used E6/E7 coding genes engineered through mutating six known oncogenic regions within E6 and E7. These mutations should disable E6 to mediate degradation of p53, and also binding to the PDZ motif inactivating the tumor suppressor protein PTPN13. Mutations in E7 (H2P, C24G, E46G and L67R) can prevent its capability to inactivate pRb and binding to Mi2 β that enhances cellular growth [25].

Since, the CD8⁺ T cells have a key immunological role in tumor elimination; we compared *in vitro* cytotoxic effect of the splenic cells isolated from the different vaccinated mice. Our findings showed a rise in CTL activity of the animal vaccinated with L1, E6, and E7 as a DNA vaccine plus NSP4. For clarification of this study, we considered that improvement of E6 and E7 oncoproteins as inducers of cellular immunity accompany with L1 and NSP4 adjuvant is possibly because of its specific epitopes. Bellone et al. (2009) found that the HPV-16 L1 DNA vaccine strongly increases the activity of vaginal CD8⁺ T cells, which are critical for the elimination of virus-infected cells [26].

In comparison with the Bellone et al. study, findings of this study revealed, that CTL response was stronger than some previous studies that can be due to reasons including the stage of tumor, the cell line, the animal model and method of CTL assay [26]. Also, pro-inflammatory cytokines may stimulate the natural killer (NK) cell, and these NK cells have key role in tumors growth suppression [27]. Even though, we didn't evaluate NK cell activity in our animal models, but the role of NK cells in tumor elimination and growth cannot be ignored. The regulation of T helper cells as effector cells is undeniable for effective immunity, therefore, enhancing all immune responses is required for considerable protection [28].

Conclusions

In summary, we developed a novel therapeutic DNA-based HPV16 vaccine, encoding a E6/E7/L1 protein and NSP4 adjuvant, which was codon-optimized for enhanced expression, and combines sequences which are secreted and ubiquitinated to induce a balanced humoral and cell-mediated immune response. We showed that this vaccine can be immunogenic and protective in the TC-1 tumor-free model and tumoric mice. This approach would be one of the promising therapeutic vaccines in HPV related cancer cases.

Declarations

Ethics approval and consent to participate

All animal experimental protocols were used according to WHO and Animal Ethics Committee of Golestan University of medical sciences approved protocols of appropriate mice care.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that this research was conducted in the absence of any relationships that could be construed as a potential conflict of interest.

Funding

This research did not receive any specific grant from the funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

Conceive and design of the experiments: A.M; data analysis: M.A, E.B; writing of the paper: E.B, S.S; performance of the experiments: S.S, M.A; Read and confirm of final version of article: A.M, E.B, M.N, S.S, M.A, A.O; Revise: E.B, A.M, M.N

Acknowledgment

The authors would like to thank Golestan University of Medical Sciences and the laboratory.

References

1. Yang A, Farmer E, Wu TC, Hung C-F. Perspectives for therapeutic HPV vaccine development. J. Biomed. Sci. 2016;23:75.
2. Lee S-J, Yang A, Wu T-C, Hung C-F. Immunotherapy for human papillomavirus-associated disease and cervical cancer: review of clinical and translational research. J. Gynecol. Oncol. 2016;27.
3. Chandra J, Dutton JL, Li B, Woo W-P, Xu Y, Tolley LK, et al. DNA vaccine encoding HPV16 oncogenes E6 and E7 induces potent cell-mediated and humoral immunity which protects in tumor challenge and drives E7-expressing skin graft rejection. J. immunotherapy (Hagerstown, Md: 1997). 2017;40:62.

4. Flatley JE, McNeir K, Balasubramani L, Tidy J, Stuart EL, Young TA, et al. Folate status and aberrant DNA methylation are associated with HPV infection and cervical pathogenesis. *Cancer Epidemiol Biomarkers Prev.* 2009;1055-9965. EPI-09-0493.
5. Castle PE, Maza M. Prophylactic HPV vaccination: past, present, and future–CORRIGENDUM. *Epidemiol. Infect.* 2016;144:2472-.
6. Klaes R, Woerner SM, Ridder R, Wentzensen N, Duerst M, Schneider A, et al. Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer Res.* 1999;59:6132-6.
7. Li L, Saade F, Petrovsky N. The future of human DNA vaccines. *J. Biotechnol.* 2012;162:171-82.
8. Liu WJ, Zhao K-N, Gao FG, Leggatt GR, Fernando GJ, Frazer IH. Polynucleotide viral vaccines: codon optimisation and ubiquitin conjugation enhances prophylactic and therapeutic efficacy. *Vaccine.* 2001;20:862-9.
9. Yang A, Jeang J, Cheng K, Cheng T, Yang B, Wu T-C, et al. Current state in the development of candidate therapeutic HPV vaccines. *Expert Rev. Vaccines.* 2016;15:989-1007.
10. Kavanagh OV, Ajami NJ, Cheng E, Ciarlet M, Guerrero RA, Zeng CQ-Y, et al. Rotavirus enterotoxin NSP4 has mucosal adjuvant properties. *Vaccine.* 2010;28:3106-11.
11. Cohen SN, Chang AC, Boyer HW, Helling RB. Construction of biologically functional bacterial plasmids in vitro. *Proc Natl Acad Sci.* 1973;70:3240-4.
12. Yang B, Yang A, Peng S, Pang X, Roden RB, Wu T-C, et al. Co-administration with DNA encoding papillomavirus capsid proteins enhances the antitumor effects generated by therapeutic HPV DNA vaccination. *Cell Biosci.* 2015;5:35.
13. Jabbar IA, Fernando GJ, Saunders N, Aldovini A, Young R, Malcolm K, et al. Immune responses induced by BCG recombinant for human papillomavirus L1 and E7 proteins. *Vaccine.* 2000;18:2444-53.
14. Greenstone HL, Nieland JD, De Visser KE, De Bruijn ML, Kimbauer R, Roden RB, et al. Chimeric papillomavirus virus-like particles elicit antitumor immunity against the E7 oncoprotein in an HPV16 tumor model. *Proc Natl Acad Sci.* 1998;95:1800-5.
15. Freyschmidt E-J, Alonso A, Hartmann G, Gissmann L. Activation of dendritic cells and induction of T cell responses by HPV 16 L1/E7 chimeric virus-like particles are enhanced by CpG ODN or sorbitol. *Antivir. Ther.* 2004;9:479-90.
16. Lenz P, Day PM, Pang Y-YS, Frye SA, Jensen PN, Lowy DR, et al. Papillomavirus-like particles induce acute activation of dendritic cells. *J Immunol.* 2001;166:5346-55.

17. Yang R, Murillo FM, Cui H, Blosser R, Uematsu S, Takeda K, et al. Papillomavirus-like particles stimulate murine bone marrow-derived dendritic cells to produce alpha interferon and Th1 immune responses via MyD88. *J. Virol.* 2004;78:11152-60.
18. Tahamtan A, Ghaemi A, Gorji A, Kalhor HR, Sajadian A, Tabarraei A, et al. Antitumor effect of therapeutic HPV DNA vaccines with chitosan-based nanodelivery systems. *J. Biomed. Sci.* 2014;21:69.
19. Sajadian A, Tabarraei A, Soleimanjahi H, Fotouhi F, Gorji A, Ghaemi A. Comparing the effect of Toll-like receptor agonist adjuvants on the efficiency of a DNA vaccine. *Arch. Virol.* 2014;159:1951-60.
20. BaghbanRahimi S, Soleimanjahi H, Mohebbi A, Ebrahimzadeh MS, Alizade L, Ghaemi A. Evaluation of DNA vaccine encoding HPV-16 E7 on Lymphocyte proliferation induction in Human Papilloma Virus-associated tumor animal models. *Jorjani Biomed J.* 2017;5:14-24.
21. DeFilippis RA, Goodwin EC, Wu L, DiMaio D. Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. *J. Virol.* 2003;77:1551-63.
22. D'Anna R, Le Buanec H, Alessandri G, Caruso A, Burny A, Gallo R, et al. Selective activation of cervical microvascular endothelial cells by human papillomavirus 16-e7 oncoprotein. *J Natl Cancer Inst.* 2001;93:1843-51.
23. Kondo K. Development of an HPV vaccine—remaining issues and perspective. *Nihon Rinsho.* 2009;67:62-8.
24. Cheung Y-K, Cheng SC-S, Sin FW-Y, Xie Y. Plasmid encoding papillomavirus Type 16 (HPV16) DNA constructed with codon optimization improved the immunogenicity against HPV infection. *Vaccine.* 2004;23:629-38.
25. Wieking BG, Vermeer DW, Spanos WC, Lee KM, Vermeer P, Lee WT, et al. A non-oncogenic HPV 16 E6/E7 vaccine enhances treatment of HPV expressing tumors. *Cancer Gene Ther.* 2012;19:667-74.
26. Bellone S, El-Sahwi K, Cocco E, Casagrande F, Cargnelutti M, Palmieri M, et al. Human papillomavirus type 16 (HPV-16) virus-like particle L1-specific CD8⁺ cytotoxic T lymphocytes (CTLs) are equally effective as E7-specific CD8⁺ CTLs in killing autologous HPV-16-positive tumor cells in cervical cancer patients: implications for L1 dendritic cell-based therapeutic vaccines. *J. Virol.* 2009;83:6779-89.
27. Raulet DH, Guerra N. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. *Nat. Rev. Immunol.* 2009;9:568-80.
28. Corthay A. How do regulatory T cells work? *Scand. J. Immunol.* 2009;70:326-36.

Figures

Figure 1
 Flow diagram of experimental design

Figure 2
 Results of tumor size measurement

Figure 3

Stimulation index given by MTT test have been demonstrated that the 6th group of study have been most affected by the DNA vaccine. (Group1 control: TC1, Group2 control: Sham)

Figure 4

The level of cytokines IL-12, IFN-gamma and IL-4 after treatment.