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DNA vaccine encoding HPV-16 E7 with mutation in L-Y-C-Y-E pRb-binding motif induces potent anti-tumor responses in mice

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A B S T R A C T

Cervical cancer is the second most common cancer among women worldwide and remains a clinical problem despite improvements in early detection and therapy. The human papillomavirus (HPV) type 16 (HPV16) E7 oncoprotein expressed in cervical carcinoma cells are considered as attractive tumor-specific antigen targets for immunotherapy. Since the transformation potential of the oncogenes, vaccination based of these oncogenes is not safe. In present study, DNA vaccine expressing the modified variant with mutation in pRb-binding motif of the HPV-16 E7 oncoprotein was generated.

A novel modified E7 gene with mutation in LYCYE motif was designed and constructed and the immunogenicity and antitumor effect of therapeutic DNA vaccines encoding the mutant and wild type of E7 gene were investigated. The L-Y-C-Y-E pRb-binding motif of E7 proteins has been involved in the immortalization and transformation of the host cell.

The results showed that the mutant and wild type HPV-16 E7 vectors expressed the desired protein. Furthermore, the immunological mechanism behind mutant E7 DNA vaccine can be attributed at least partially to increased cytotoxic T lymphocyte, accompanied by the up-regulation of Th1-cytokine IFN-γ and TNF-β and down-regulation of Th3-cytokine TGF-β. Immunized mice with mutant plasmid demonstrated significantly stronger cell immune responses and higher levels of tumor protection than wild-type E7 DNA vaccine.

The results exhibit that modified E7 DNA vaccine may be a promising candidate for development of therapeutic vaccine against HPV-16 cancers.

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1. Introduction

Cervical cancer is the second most common cause of women deaths in all over the world. It would result in death of approximately 250,000–290,000 women each year globally, particularly in developing countries. Clinical, molecular and epidemiological investigations have identified human papilloma virus (HPV) as the major cause of cervical cancer (Trottier and Burchell, 2009).

Virtually all cervical cancers (about 99%) contain the genes of high-risk HPVs, most commonly types 16, 18, 31, and 45. In addition, HPV may play a role in certain carcinomas of the head and neck region and perhaps other cancers (Cubie, 2013). Therefore, it is necessary to develop therapeutic vaccines to reduce infection

Abbreviations: HPV, human papilloma virus; PHA, phytohemaglotinin; APC, antigen-presenting cell; CTL, cytolytic T lymphocyte; Rb, retinoblastoma; IFN-γ, interferon γ; IL-4, interleukin 4; TNF, tumor necrosis factor; TGF-β, transforming growth factor beta; PVDF, polyvinylidene difluoride membranes; LDH, lactate dehydrogenase; MTT, 3[4,5-dimethylthiazol-2-ll]-2,5-diphenyltetrazolium bromide, thiazolyl-blue; DMSO, dimethyl sulfoxide; OD, optical density; FBS, fetal bovine serum; CHO, Chinese hamster ovary; RPMI, 1640 Roswell Park Memorial Institute (name of the medium); Th, T helper.

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or HPV-related cancers especially cervical cancer (Elfstrom et al., 2014; Tran et al., 2014).

As the late proteins L1 and L2 are not detected in cervical cancer or infected basal cells, most therapeutic vaccines target the HPV early proteins such as E6 and E7. These oncogenic proteins are critical to the induction and maintenance of cellular transformation and are co-expressed in the majority of HPV-containing carcinomas (Morrow et al., 2013).

When a cell is infected with HPV, the E7 abrogate retinoblastoma (Rb) protein function, preventing it from interacting with E2F. Because E2F is now free, it promotes further rounds of cell division (Ghittoni et al., 2010). E7 also alter cytokine expression pattern, resulting in immune evasion (Sasagawa et al., 2012).

DNA vaccines targeting the E7 antigen offer a potentially effective procedure in HPV therapeutic vaccine development against E7-expressing tumors. DNA vaccines represent a promising strategy for generating antigen-specific immunotherapy because of their simplicity, stability, safety, and capacity for repeated administration (Li et al., 2012).

Although some experts believe that DNA vaccines are safer than live recombinant vaccines, others have raised concerns that the injected DNA might become integrated into the host genome, potentially inactivating tumor suppressor genes or activating oncogenes (Peng et al., 2006). DNA vaccines encoding E7 oncoprotein can either stably integrate into the genome or are maintained in an episomal form allowing for extended expression of HPV antigens (Eiben et al., 2003). In order to prevent vaccination-induced cellular transformation, modification in the pRb binding sites is necessary to eliminate the potential for oncogenic transformation while preserving critical epitopes (Ohlschlager et al., 2006).

The HPV16 E7 protein represents a zinc finger-binding phosphoprotein with two Cys-X-X-Cys domains composed of 98 amino acids. HPV16 E7 protein binds Rb through an L-Y-C-Y-E (conserved region 1; aa 21–26) motif (Cassetti et al., 2004; Munger et al., 2001). It has been shown that the transformation potential of the E7 oncoprotein is mainly localized in its pRb binding site (Smahel et al., 2001). As this interaction is probably required for carcinogenic progression in human patients, then therapeutic blockade of this activity could provide new treatment strategies in cervical carcinoma (Pang et al., 2013).

Previous study have demonstrated that mutation affecting only Cys of the repeats, which are conserved between different HPV E7 proteins, severely reduced the transforming activity but did not totally destroy it (Cassetti et al., 2004; Shi et al., 1999; Smahel et al., 2001). In order to design an E7 DNA vaccine with reduced transformation capacity and increased stability, three point mutations were introduced into the L-Y-C-Y-E pRb-binding motif (23 Tyr to Gly, 24 Cys to Gly, 25 Tyr to Gly) and mutated E7 gene was designed as DNA vaccine and administrated in tumor cell expressing HPV16 antigens. The immunogenicity and antitumor effect of the mutated vaccine was compared with wild E7 DNA vaccine.

2. Materials and methods

2.1. Mice

6 ± 8-week-old female C57BL/6 mice were purchased from the Pasteur Institute (Karaj, Iran) and kept in the laboratory animal facility of Golestan University of medical science. All animals were fed with enough food and water to pass adaptation period, and treated with 6.00–18.00-h light/dark cycle. Approved protocols were applied to all animal experiments with consideration of recommendations for the accurate use and care of laboratory animals by the ethical commission of Golestan University.

2.2. Cell lines

TC-1, (Part of the Johns Hopkins Special Collection) was derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV16 E6 and E7 and activated c-Ha-ras oncogene. TC-1 cell line which is HPV-16 $E7^+$ was used as a tumor model in an H-2b murine system. TC-1 and CHO Chinese hamster ovary cell lines were grown in Roswell Park Memorial Institute medium (RPMI 1640) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin/streptomycin 50 U/ml, 2 mM glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, and G418 0.4 mg/ml at 37 °C with 5% CO_2.

2.3. Construction of the recombinant vector

In the study, pcDNA3 plasmids which contain the mutant and wild type HPV-16 E7 DNA vaccines (23 Tyr to Gly, 24 Cys to Gly, 25 Tyr to Gly) under the control of the cytomegalovirus immediate-early promoter/enhancer (CMV-IEPE) were used.

The generation of pcDNA3-E7 has been described previously. Plasmid constructs were confirmed by DNA sequencing and expression. The immunogenicity of the construct had been evaluated in the previous experiment (Ghaemi et al., 2011).

The mutant HPV16 E7 gene was chemically synthesized by MWG Biotech (Ebersberg, Germany) and provided in a pEX-A vector ready for excision via the EcoRI and XhoI restriction sites at the 5′ and 3′ ends, respectively. This sequence contained three substitutions at LYCYE motif, mutations at positions 23Y, 24C and 25Y of this motif abolish its RB-binding capability. The resulting gene was sequenced to ensure that only the desired change had been introduced, and then subcloned into EcoRI/XhoI site of the eukaryotic expression vector pcDNA3.1 (Invitrogen, San Diego, CA, USA).

2.4. Gene expression analysis

CHO cells were cultured in RPMI 1640 containing 10% FBS. Plasmids were transfected by Lipofectamine 2000 (Invitrogen, San Diego, CA, USA), and cells were harvested between 24 and 48 h after transfection. Cellular protein from transfected CHO cells were extracted using the lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, and 0.5% Nonidet P-40). Proteins were separated by sodium dodecyl sulfate (SDS)/PAGE and analyzed by Western blotting with the Anti-E7 antibody.

Separated proteins were blotted onto polyvinylidene difluoride membranes (PVDF) (Roche, Germany), and treated with the Anti-HPV-16 E7 Polyclonal Antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by detection with goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma, St Louis, MO, USA) in secondary antibody solution. Color was developed by incubating the membrane in alkaline phosphate buffer containing Tetramethylbenzidine (TMB) Substrate Solution.

2.5. Tumor challenge and Immunizations

In immunotherapeutic experiments, female mice of 6–8 weeks ($n = 10$) were first inoculated by subcutaneous injection in the right flank with 3×10^5 TC-1 cells. One week after tumor cell transplantation, the mice were immunized with 100 μg DNA vaccine encoding mutant and wild type HPV-16 E7 genes, pcDNA3 and PBS via intramuscular injection. The mice received 2 boosts with the same regimen 1 and 2 weeks later. Tumor growth was monitored and estimated according to Carlsson's formula. Hence, the largest (a) and the smallest (b) superficial diameters of the tumor were measured twice a week and then the volume (V) of the tumor was calculated ($V = a \times b \times b/2$) (Ghaemi et al., 2010). Statistical analysis

was performed using Student's t test. All values were expressed as mean ± SD.

2.6. Cytotoxicity assay

One week after last immunization, the mice (three mice of each group) were sacrificed and their splenocytes were isolated. For each sample obtained from individual mice, single-cell suspensions of mononuclear cells (used as the effector cells) were cultured in RPMI 1640 medium with washed EL4 target cells (a mouse lymphoma cell line derived from C57BL/6 (MHC-H2b); ATCC TIB-39, from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran)) at various effector-to-target cell (E/T) ratios (25:1, 50:1, 100:1) and in 96-well flat-bottom plates for 4 h in phenol red-free RPMI 1640 containing 3% FBS.

For preparation of the target cells, EL4 cells were stimulated with 4×10^5 TC-1 cells treated previously with mitomycin C (30 μg/ml for 3 h) and then incubated for 4 h. After centrifugation, the supernatants (50 μl/well) were transferred into the 96-well flat-bottom plates, and lysis of target cells were determined by measuring lactate dehydrogenase (LDH) release using a LDH cytotoxicity detection kit according to the procedures stated by the manufacturer (Takara Company, Shiga, Japan). Several controls were used for the cytotoxicity assay.

The 'target maximum' was the total LDH released from the target cells, and all EL4 cells were lysed by medium containing 1% Triton X-100. The 'target spontaneous' was the natural release of LDH from the target cells, which was obtained by adding EL4 cells only to the assay medium. The 'T cell control' was used to measure the natural release of LDH from T cells and was obtained by adding the different ratios of T cells only to the assay medium.

For all samples, including the controls, the assay was performed in triplicate. The LDH-mediated conversion of tetrazolium salt into a red formazan product was measured at 490 nm after incubation at room temperature for 30 min. The percentage of specific cytolysis was determined by the following formula:

$$\text{Cytotoxicity} = \left[(\text{experimental value} - \text{effector spontaneous}) - \frac{\text{low control}}{\text{high control} - \text{low control}}\right] \times 100$$

2.7. Lymphocyte proliferation assay

One week after third immunization, a single-cell suspension of mononuclear cells obtained from immunized mice was used for the lymphocyte proliferation assay (LPA). Briefly, the suspension of isolated spleen cells was treated with lysis buffer (0.15 MNH_4Cl; 1 m $MKHCO_3$; 0.1 mM Na_2EDTA; pH 7.2) in order to clear red blood cells. In 96-well flat-bottom culture plates (Orange Scientific, Waterloo, Belgium), 2×10^5 cells per well were cultured. The preparations were cultured in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol and 0.1% penicillin/streptomycin, and incubated in the Presence of 4×10^5 TC-1 cells treated previously with Mitomycin C (30 g/ml for 3 h) per well at 37° in 5% CO_2. Five μg/ml T cell mitogen phytohemagglutinin (PHA) (Sigma Chemicals, St. Louis, MO, USA) was used as the positive control. After 3 days, 5 μg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemicals, St. Louis, MO, USA) was added to each well and incubated for 5 h at 37° in 5% CO_2. DMSO (100 μl) was added to dissolve formed formazan crystals. Then, the plates were read at 540 nm, and the results expressed as a stimulation index (SI). The SI was determined as follows:

$$\text{SI} = \frac{\text{OD unstimulated culture}}{\text{OD stimulated culture}}$$

All tests were performed in triplicate for each mouse.

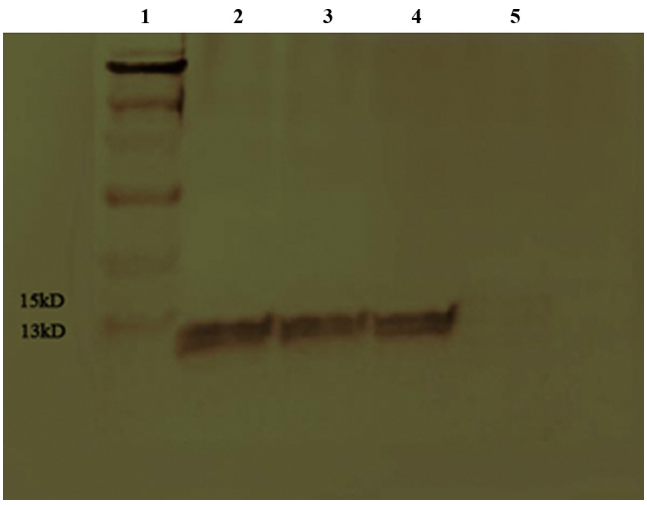

Fig. 1. Western blot analysis of mutant and wild types of E7. CHO cells were transfected with the various plasmids, and proteins in cell lysates were resolved by SDS–PAGE and analyzed by Western blot with anti-E7 antibody. Lanes are labeled as follows: 1: Protein marker, 2: TC-1 lysate, 3: E7 Wild, 4: Mutant E7, 5: pcDNA3.

2.8. Cytokine secretion assay

One week after the third immunization, mononuclear cells from spleens of immunized mice at a concentration of 2×10^6 cells per well were incubated in 24-well plates for 3 days in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 1% HEPES, 0.1% 2-mercaptoethanol and 0.1% penicillin/streptomycin, and pulsed with 4×10^5 TC-1 cells treated previously with mitomycin C (30 μg/ml for 3 h) at 37° in 5% CO_2.

The supernatants were collected and assayed for the presence of IFN-γ, IL-4, TNF-α and TGF-β, using commercially available sandwich-based ELISA kits (eBioscience, San Diego, USA) following the manufacturer's instruction. All tests were performed in triplicate for each mouse.

2.9. Statistical analysis

To compare results between the different groups, a one-way ANOVA was used. The statistical software SPSS version 16.0 was utilized for statistical analyses. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Construction of mutant HPV-16 E7 DNA vaccine

The accuracy of inserted E7 fragment was confirmed by restriction enzyme analysis and sequencing. Western blots were performed for protein expression analysis. At 48 h post transfection, cell lysates were analyzed by western blot using anti-E7 antibody. As shown in Fig. 1, a very strong band of approximately 13 kDa, corresponding to E7, was observed in cells transfected with pcDNA3-mutant E7 (lane 4). This E7 band was also visible in cells transfected with pcDNA3-wild type E7 (lane 3). Cell lysate from TC-1 was used as positive control (lanes 2). In contrast, transfected CHO Cell lysate with pcDNA3 used as negative control (lanes 5).

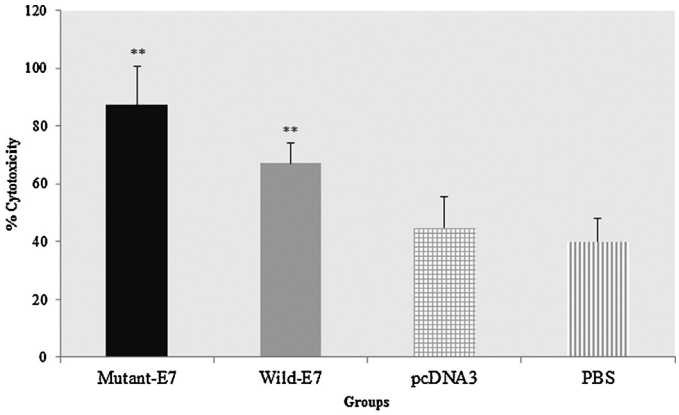

Fig. 2. Strong CTL effects induced by mutant HPV-16 E7 DNA vaccine. Mice ($n = 3$) were immunized thrice with the mutant and wild type HPV-16 E7 DNA vaccines with a one-week interval. The LDH release rate from the target cell EL4 was assessed as the E7-specifc cytolytic activity of effector splenocytes and expressed as cytotoxicity percentage ± SD. Results are representative of at least three independent experiments. **Indicates statistically significant difference between the marked groups as determined by one-way ANOVA ($P < 0.01$). The graph also shows the statistical significant differences between the mutant and wild type HPV-16 E7 DNA vaccines with PBS and pcDNA3 control groups ($P < 0.05$).

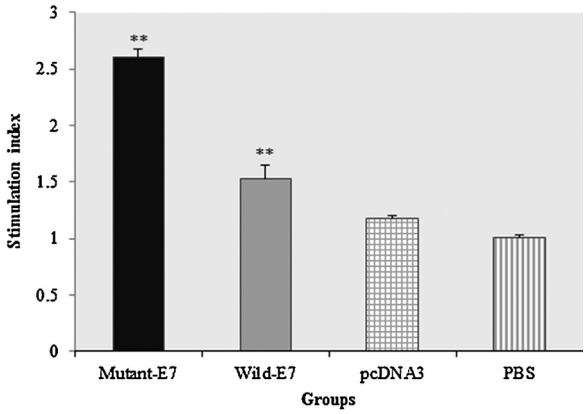

Fig. 3. Significant cell proliferation stimulated by mutant HPV-16 E7 DNA vaccine. Mice ($n = 3$) were immunized thrice with the mutant and wild type HPV-16 E7 DNA vaccines with a one-week interval. One week after the last immunization, splenocytes were harvested from each mouse and re-stimulated with the E7 antigen in vitro and assessed for proliferation capacity by the MTT assay. Each experiment was repeated three times and data shown are mean ± SD. Results are representative of three independent experiments. **Indicates statistically significant difference between the marked groups as determined by one-way ANOVA ($P < 0.01$). The graph also shows the statistical significant differences between the mutant HPV-16 E7 DNA vaccine with PBS and pcDNA3 control groups ($P < 0.05$).

3.2. CTL assay

DNA immunization with E7 has been shown to induce CTL responses (Ghaemi et al., 2011). Herein it was investigated whether CTL responses could be induced by immunization with plasmid DNA encoding a modified E7. The CTL response in immunized mice was examined in present study by the LDH release assay in 96-well plates. The mice were immunized three times, and the CTL activity was measured as described before. Cytolytic activity was most effective at effector: target ratio of 100:1 (100:1 E/T), with maximal cytotoxic responses to EL4 target cells.

As shown in Fig. 2, lymphocytes in vaccinated mice with mutant HPV-16 E7 (87.21 ± 13.2%) and wild type HPV-16 E7 (67.17 ± 6.9%) at 100:1 E/T ratio had a significantly increased specific cytolytic activity when compared to that of pcDNA3 (44.7 ± 10.9%) and PBS (40.03 ± 8%) as negative control groups ($P < 0.05$). According to these results, they showed that the immunized mice with the mutant E7 DNA vaccine induced higher levels of CTL activity than those of the wild E7 DNA vaccine ($P < 0.01$). The results from three representative experiments are expressed as the percentage of specific lysis at a 100:1 effector to target cell ratio.

3.3. Lymphocyte proliferation assay

Since lymphocyte proliferative responses are generally considered as a measure of cell-mediated immunity, HPV E7 antigen-specific lymphocyte proliferation was evaluated by MTT assay.

Mice were immunized three times with the DNA vaccines at one-week intervals: mutant E7, wild E7, pcDNA3, or PBS. One week after the last immunization, the splenocytes from the immunized mice were harvested and re-stimulated in vitro with E7 for the lymphocyte proliferation assay. As shown in Fig. 3, the splenocytes from mice inoculated with mutant HPV-16 E7 showed significantly higher lymphocyte proliferation than any of the other groups ($p < 0.05$) as indicated by the proliferation index >2.5. In contrast, neither wild E7, pcDNA3, nor PBS could induce remarkable cell proliferation, as indicated by the respective indexes.

3.4. Cytokine assay

To investigate the potential mechanisms responsible for the observed therapeutic efficacy of mutant E7 DNA vaccine on TC-1 tumor, the proportion of different cytokines between mutant and wild type HPV-16 E7 DNA vaccines-treated, and control groups were compared. Therefore, the levels of Th1-cytokine IFN-γ and TNF-α, Th2-cytokine IL-4, and Th3-cytokine TGF-β in the supernatant of cultures from splenocytes stimulated with E7 antigen were examined. As shown in Table 1, splenocytes taken from immunized mice which received mutant HPV-16 E7 vaccine produced higher levels of IFN-γ in comparison to any of the other groups ($p < 0.05$) including the wild E7 DNA group, while concentrations of the Th3-cytokine TGF-β (anti-inflammatory cytokine) in mutant HPV-16 E7 DNA vaccine group were significantly lower than those in any of the other groups ($P < 0.05$). Of note, the level of another of the Th1-cytokine TNF-α in both mutant HPV-16 E7 vaccine and wild HPV-16 E7 vaccine groups were significantly higher than those in the negative control groups ($P < 0.05$), but mutant HPV-16 E7 vaccine and wild HPV-16 E7 vaccine groups showed no significant difference with each other in TNF-α level.

It was found that concentrations of Th2-cytokine (IL-4) in mutant HPV-16 E7 vaccine was not significantly different among the groups ($P > 0.05$), although there was a slight increase in mutant HPV-16 E7 vaccine group compared to the negative controls. Together, these observations suggest that mutant HPV-16 E7 vaccine, indeed shifted toward a predominant Th1 profile.

Table 1
IFN-γ, IL-4, TGF-β and TNF-α levels in culture supernatants of splenocytes from immunized mice one week after last vaccine administration ($n = 3$, mean ± S.D.).

Vaccine	IFN-γ	IL-4	TGF-β	TNF-α
Mutant E7	238.5 ± 13.5[a,b]	143.05 ± 8.4	110.3 ± 4.4[a,b]	357.6 ± 5.6[a]
Wild E7	152.3 ± 12.3	119.6 ± 11.6	176.7 ± 7.3	266 ± 6.3[a]
pcDNA3	71.4 ± 3.4	70.3 ± 2.3	314.6 ± 2.3	121.5 ± 3.4
PBS	56.3 ± 8.4	53 ± 5.5	307.6 ± 5.5	68.5 ± 6.2

Results are representative of three independent experiments.
[a] Shows the statistical significant differences between two DNA vaccine with PBS and pcDNA3 control groups as determined by one-way ANOVA ($P < 0.05$).
[b] Shows the statistical significant differences between mutant E7 with Wild E7 as determined by one-way ANOVA ($P < 0.05$).

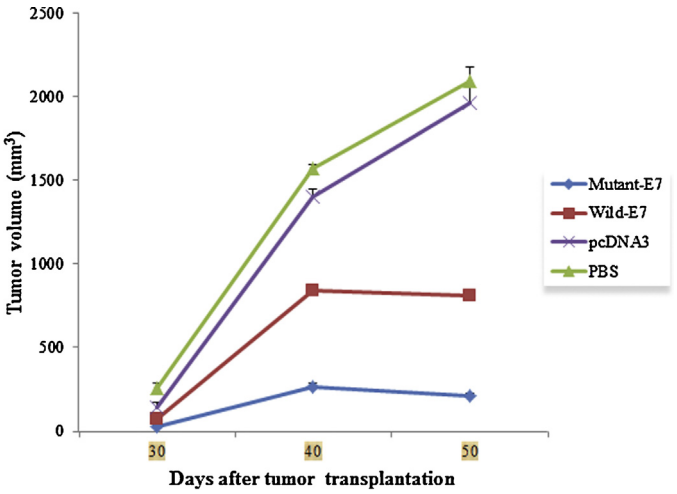

Fig. 4. Graphical representation of the tumor volume in mice challenged with 2×10^5 TC-1 tumor cells subcutaneously. Mice were then treated with the HPV-16 mutant E7 and the HPV-16 wild E7 vaccine as described in Materials and Methods. Mice were monitored twice a week for tumor growth. Line and scatter plot graphs depicting the tumor volume (in mm^3) are presented. Tumor treatment experiments were performed three times to generate reproducible data.

3.5. Antitumor effect of therapeutic DNA vaccine

To determine whether the mutant HPV-16 E7 vaccine could provide protection against tumors in vaccinated mice, C57BL/6 mice were immunized thrice with the mutant HPV-16 E7 vaccine and the wild HPV-16 E7 vaccine using the same dose and vaccination regimen for each construct. After the last vaccination, the mice were challenged subcutaneously with HPV-16 tumor cell model, TC-1. The tumors were measured twice a week once they became palpable.

Tumors grew rapidly in mice inoculated with pcDNA3 and PBS, whereas tumor growth was retarded significantly in mice treated with DNA vaccines expressing the wild or mutant E7 protein. As shown in Fig. 4, at 30, 40 and 50 days after vaccination, mean tumor volumes were statistically different in the DNA vaccine groups compared to negative control groups ($p < 0.001$, one-way ANOVA). Tumor volumes were 2.7-fold less in the mutant HPV-16 E7 vaccine group 30 days after immunization than the wild HPV-16 E7 vaccine group. At 40 and 50 days after vaccination, the average tumor volumes of the mutant E7-vaccinated animals were significantly 3.2- and 3.8-fold less ($P < 0.001$) than in those vaccinated with the wild HPV-16 E7 vaccine. The mice vaccinated with the wild HPV-16 E7 vaccine had the smallest tumor volumes 50 days after TC-1 tumor challenge ($p < 0.001$, one-way ANOVA). Similar results were obtained in three independent experiments with seven mice per group.

These results indicate that vaccinations with mutant E7 generate potent immune responses and anti-tumor effects.

4. Discussion

The human HPV E7 protein is expressed selectively in cervical cancer cells and thus is considered the prime target for cell mediated immunotherapy (Hung et al., 2007). Therefore, the goal of immunization against HPV-induced malignancies is to boost cellular immune system that eliminates cancerous cells. Several E7-specific therapeutic vaccines are being developed currently (Morrow et al., 2013; Tomson et al., 2004). Among them, DNA vaccination has emerged as an attractive strategy for generating antigen-specific immunotherapy because of low production costs, overall stability and ease of storage (Moniz et al., 2003). However, because the E7 protein is known to have transforming activity, this DNA vaccine might have oncogenic potential. Mutation in the E7 protein, which abrogates the transforming activity, helps to ensure the safety of the DNA vaccine (Ohlschlager et al., 2006). The present study demonstrated that a therapeutic DNA vaccine encoding mutant E7 antigen is able to induce significantly a cellular immune response and reduce tumor volume against H-2b tumor cell line, TC-1 (containing the HPV16 E6, E7) compared to the DNA vaccine encoding wild E7 gene.

Selecting optimized mutations that interfere with binding to the host cell Rb protein is important to produce an efficient mutated E7 vaccine. Previous studies have exhibited those amino acids in second conserved region (CR2) shared by most E7 protein form tight complexes with small pocket of retinoblastoma tumor suppressor protein, pRb (Ghittoni et al., 2010; Singh et al., 2005). The Leu-Tyr-Cys-Tyr-Glu (L-Y-C-Y-E) motif (aa 22–26) comprises the core for pRb pocket binding (Comerford et al., 1991). The LYCYE pRb-binding motif of high risk HPV E7 proteins has been implicated in the immortalization and transformation of the host cell (Ledl et al., 2005; Narechania et al., 2004).

Using various LYCYE sequences and mutant peptides from E7, it has been demonstrated that this interaction is complex and involves residues other than Cys. Barbosa et al. have demonstrated that mutation at Cys 24 induces partial loss of affinity for pRb (Barbosa et al., 1990). Recent study has also showed that single amino acid substitution may not be sufficient for inactivation of the pRb binding to LYCYE sequences (Singh et al., 2005). It was also found that the E7 gene can be genetically modified to abrogate its binding to pRb by generating three amino acid substitutions (Asp 21, Cys 24, Glu 26), thus reducing its transforming potential. The resulting E7GGG protein can also increase its immunogenicity and therapeutic efficacy (Smahel et al., 2001). It has been shown that vaccination with integrase defective lentiviral vector encoding an E7GGG form of HPV16 E7 protein induced a potent and persistent E7-specific T cell response (Grasso et al., 2013). It has been also reported that the mutations in two zinc-binding motifs or pRb-binding sites can diminish the transformation ability of E7 protein (Li et al., 2007).

Among other residues, Tyr residue at aa 23 is highly conserved among different papillomaviruses and mutation of these residues result in considerably weaker pRb binding. Tyr residue at aa 25 also participates in forming precise conformation of Rb-binding domain (Jones et al., 1990, 1992). Therefore, utilizing the E6- and E7-expressing murine tumor cell line TC-1 as a model of cervical carcinoma, for the first time a modified E7 gene with mutations in aa 23–25 was designed and constructed (Tyr-Cys-Tyr) and the wild-type residues with glycine were substituted, then the immunogenicity and antitumor immunity of DNA vaccine encoding the resulting mutant in tumor mice model was evaluated.

Cell mediated immune response play an important role in antitumor immunity. It has been demonstrated that immunization with plasmid encoding E7 gene activates CTL responses and induces tumor protection in vivo (Ghaemi et al., 2011; Morrow et al., 2013). In the present study, immunization with mutant E7 in LYCYE pRb-binding motif also elicited specific CTLs, which was demonstrated by the fact that these CTLs lysed specifically target cells expressing E6 and E7. The similar CTL response of the mutant and wild E7 vaccines are likely due to the H-2Db RAHYNIVTF (49–57) epitope, the only E7 CTL antigenic epitope described for the C57BL/6 mouse (Santana et al., 2013). The lymphocyte proliferation and interferon γ assay shown in Figs. 3 and 4, in which the splenocytes are stimulated with the specific antigen, is showing approximately higher response in the mice vaccinated with the mutant E7 than those vaccinated with the wild type one.

The antitumor efficacy of mutant E7 was also compared with that of E7. During the tumor challenge experiment, E7-vaccinated

mice rejected the TC-1 cell challenge, as observed previously (Ghaemi et al., 2010), but mice treated with the mutant E7 had reduced significantly tumor volumes when compared to mice treated with the wild E7 vaccine. The result demonstrated that mutant E7 might induce stronger antitumor immune responses than E7.

Although the precise mechanism by which DNA vaccine encoding mutant E7 exerts its antitumor effect is not yet fully understood, current findings are highly suggestive that it may act by increasing the level of cytotoxic T lymphocytes and lymphocyte proliferation, inducing a shift in Th1/Th2 balance toward Th1-dominant, accompanied by the up-regulation of Th1-cytokine IFN-γ and TNF-β and down-regulation of Th3-cytokine TGF-β Indeed. TGF-β, which is abundantly secreted by regulatory T lymphocytes and tumor cells, has been found to promote invasion and metastases for many cancers and impair tumor-specific T cells (Achyut and Yang, 2011). The existence of regulatory T lymphocytes able to limit the immune response in a specific form has been established, specially inhibiting the proliferation and activity of CD4+ and CD8+ effector T lymphocytes (Lopez et al., 2006). The observations exhibit that reduction of TGF-β levels downregulates regulatory T lymphocytes by the mutant E7 vaccine and enhances antitumor activity.

In agreement with the present study, Radaelli et al., showed enhanced immune response to recombinant fowlpox expressing the non-oncogenic HPV-16 E7GGG protein than its wild type (Radaelli et al., 2012). Chen et al. also have found that de-oncogenic versions of the E7 augmented the immunogenicity and antitumor effects compared with wild-type E7 (Chen et al., 2014). It has been shown that higher antigen presentations might stimulate stronger CTL responses. Shi et al., showed that mutations in two zinc binding motifs of E7 leads to rapid degradation of the protein (Shi et al., 1999) and other study has shown that an unstable protein has greater potential to generate CTL responses than a stable one (Zhang et al., 2004). Wong et al. found that targeting of antigens for rapid proteasomal degradation could enhance their processing and presentation in association with MHC class I (Wong et al., 2004). These observations may explain in part the better protective effect of mutant E7 in the present study.

In summary, the current results suggests that modified E7 gene with mutations in aa 23–25 leads to the increased presentation of E7, resulting to the enhancement of DNA vaccine potency by generating stronger CD8+ T cell immune responses as well as more potent therapeutic antitumor effects. Therefore, mutant E7 DNA vaccine might be a promising novel therapeutic approach to control HPV-16-associated tumor formation and to reduce cervical cancer.

Conflict of interest

All the authors have no conflicting interests.

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