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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.

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