Original Article

Construction a DNA Vaccine Containing Human Papillomavirus Type 16 Early Genes as a Potential Vaccine for Cervical Cancer Prevention and Therapy

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ABSTRACT

Background and Objectives: **Some of the human papillomaviruses (HPVs) can infect genital tracts and are sometimes associated with anogenital tract cancers. HPVs induced cervical cancers through the expression of E6 and E7 genes by inactivating the tumor suppressor proteins. In this study, E6 and E7 genes were chosen in order to construct an expression vector which is able to express target proteins.**

Patients and Methods: **This experimental investigation was performed in Virology Department of Tarbiat Modares University. An expression vector containing human papillomavirus type 16 E6 and E7 genes was constructed. The accuracy of the plasmid was confirmed by polymerase chain reaction (PCR) and restriction enzyme analysis. The construct was transfected into the eukaryotic cells and its ability for protein production was confirmed by Western blotting.**

Results: **The colonies containing desired plasmid have the fragment about 995 bp. For confirming the ability of the construct for protein production in eukaryotic cells, Western blotting was done using the lyses-cells as antigen and they showed the desired bands using monoclonal antibodies.**

Conclusion: **The designed vector can consider as a based vaccine for construction a therapeutic vaccine in suitable vectors for gene therapy in order to administration in Iranian patients with cervical cancer.**

Keywords: Human papillomavirus 16, HPV Vaccines, Recombinant Proteins

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Introduction

Cervical cancer is the second most common cancer's deaths in women world wide and about 500,000 women develop the cervical cancer tumors and 200,000 women died each year in the world (1). There is a very good correlation between oncogenic HPVs and the development of ano-genital intraepithelial neoplasia conditions. In addition, there are many strong evidence which the induction a strong cell-mediated immunity against HPV antigens, lead to the elimination of infected cells. There are several published data which have reported the existence of HPV DNAs in tumour cells in more than 90% of patients (2,3). HPV types 16 and 18 are the common members of the high risk HPV group and their DNA can integrate into the host cell genome (4). The HPV-16 can be detected in about half of all invasive cervical cancers and it is the most prevalent high risk HPV in cervical cancer specimens among Iranian women (5,6). The high rates of infection with HPV genotypes in sexually active Iranian women make the preventive and therapeutic vaccine essential for the patients with proved dysplasia or cervical cancer. HPV-16 is an oncogenic DNA virus which encodes six early and two late genes. HPV16 E6 and E7 proteins are the major oncoproteins and they can disturb the control of the cell cycle by their interaction with cellular proteins that are important for progression through the G1 restriction point (7;8). The E6 protein binds to the p53 tumor suppressor protein and degrades the protein and interferes with both cell cycle control and the apoptotic pathway (9,10). The E7 protein shows multiple functions including decreases the transcriptional activity of p53 (11). Therefore, published data suggest that the E6 and E7 proteins play an important role in HPV-associated malignancy. It is important to note that the E6 and E7 proteins are expressed in all tumor tissues; therefore, vaccines for immunotherapy targeting E6 and E7 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies.

DNA vaccination is a useful mean of expressing specific antigens for generation humoral and cellular immune responses. In addition, plasmid DNA is safe and it has low immunogenicity, so it can be repeatedly administered. The DNA vaccine preparation is easy especially in large scale with high purity (12-14). Thus, DNA vaccines provide a powerful instrument for cancer prevention and therapy.

In this study, we constructed a potential DNA vaccine containing HPV16 E6 and E7 open reading frames and the expression of the recombinant proteins were confirmed by RT-PCR and Western blotting. This construct can use as a potential DNA vaccine for HPV16 cervical cancer therapy.

Material and Methods

Plasmid DNA construction and preparation: This experimental study was done in Virology Department of Tarbiat Modares University from 2006 to 2007. DNA fragment encoding human papillomavirus type 16 E6 and E7 was obtained from pTZ57R-E6 (15) and pTZ57R-E7 (16), respectively. For generation of E7 expression plasmid (pcDNA3-E7), the E7 gene was subcloned from pTZ57R-E7 into the unique EcoR I and Xba I cloning sites of the pcDNA3 expression vector (Invitrogen, UK), down-stream of the cytomegalovirus promoter. For the generation of E6-E7 DNA vaccine (pcDNA3-E6/E7), the E6 was subcloned from pTZ57R-E6 into the unique Hind III and BamH I cloning site of the pcDNA3-E7 expression vector at the 5' end of E7 gene.

Confirming the accuracy of constructed plasmid: Colonies containing the constructed plasmid were obtained by transformation of Ecoli DH5α bacteria (Invitrogen Company, UK) as host. The colonies containing target clone were selected by ampicillin resistance and the accuracy of constructed plasmid was confirm by colony-PCR and restriction enzyme analysis. Colony-PCR was performed using E6 forward (5' TAATCGAAGCTTAAAACTAAGGG CGT 3') and E7 reverse (5' GATCTGCCTCTAGAGATTAT GGTTTCTGA 3') primers. The colonies used directly as template in colony-PCR. PCR mixture consisted of 50 pmol each primers, 1.5 mM MgCl2, 0.2 mM each dNTP, 1 U Taq polythermase (Cinagen Company, Iran) in a total reaction volume of 15 µl. Amplification was carried out for 35 cycles (94°C for 30 s, 64°C for 30 s, 72°C for 45 s) after an initial denaturation step in 94°C for 5 min, on a Techne

Thermal Cycler (Bio Rad Company, UK). The cycles were followed by a 5 min extension at 72°C and the PCR product was visualized on a 1.5% agarose gel by ethidium bromide staining.

Restriction enzyme analysis also used for confirming. Firstly, the colonies those were positive in colony-PCR selected, propagated and purified. The restriction enzymes Hind III and Xba I (Roche Company, Germany) were used for enzyme analysis. The confirmed construct was prepared in large scale and it was used in eukaryotic transfection system.

Cell line: CHO cells (as negative control) and CHO/E6-E7 transfected cells were cultured in RPMI 1640 (Gibco Company, Germany) supplemented with 10% fetal calf serum (FCS) (Gibco Company, Germany), 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 1 mM sodium pyrovate and they were grown at 37°C and 5% CO2.

Analysis of eukaryotic protein expression: To monitor expression of E6-E7 recombinant protein, 10^6 CHO cells were seeded into a 6-wells micro-plate and incubated overnight in complete medium without antibiotic. When they were 70% confluent, they were used for transfection. CHO cells were transfected with pcDNA3/E6-E7 using calcium-phosphate protocol (17). At 48 h after transfection, the cells were washed with PBS and then were used for RT-PCR and Western blot analysis.

RT-PCR and Western blot analysis: Protein expression in transfected cells was firstly determined by RT-PCR using the specific primers. In brief, at 48 h after transfection, the cells were washed with PBS and total cellular RNA was purified from the cells using RNX Plus kit (Cinagen Company, Iran). The contaminating DNA was removed by DNase treatment and the extracted RNA was used in RT-PCR (cDNA synthesis was performed on 1 µg total RNA with Oligo-dT primers at 37ºC and PCR was performed on the cDNA by the E6 forward and E7 reverse primers as described before).

For Western blotting analysis, the cell extracts were prepared from both un-transfected and transfected CHO cells 48 h following induction with pcDNA3/E6-E7. Total cell lysates were subjected to 5 times freezing and thawing and then sonication (60 HZ, 0.5 amplitude) was done for the cells. PMSF (phenylmethylsulfonyl fluoride, Sigma Company, 1 mM) was added to cell lysate to inactivate proteinase. The samples were loaded on a 12% SDS-PAGE gel after boiling for 10 min and transferred to PVDF Western blotting membrane (Roche Company, Germany) by electrophoresis for 1 h at 90 V. Membranes were blocked in 3% BSA in PBS for 1 hour. E6 and E7 proteins were detected using a monoclonal mouse anti-E6 and anti-E7 antibodies (Abcam Company, UK), respectively, and specific bands were visualized with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G antibody (TEBSUN Company, Iran) and stained by reaction with diaminobenzidine (DAB) substrate (Biogen Company, Iran).

Results

The correctness of desired plasmid was confirmed by colony-PCR using E6 forward and E7 reverse primers. As shown in Fig. 1, the colonies containing desired plasmid were positive and their fragment was about 995bp. The colonies that were positive on colony-PCR were propagated and their plasmids were purified. Restriction enzyme analysis using Hind III and Xba I enzymes was also performed for confirming. As it is showed in Fig. 2, using two above restriction enzymes, the fragment about 995 bp came out. The confirmed construct was propagated and used for transfection study. CHO cells were used for performing transfection.

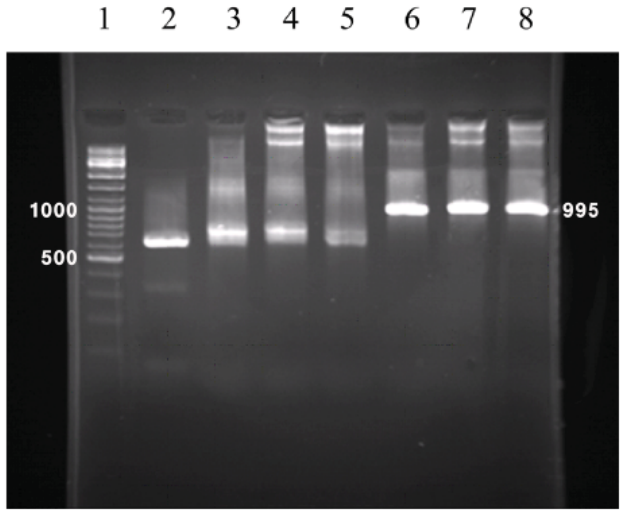

Fig. 1: Colony-PCR for colonies using E6 forward and E7 reverse primers. The lane umbers 6, 7 and 8 showed desired fragment (995 bp) of HPV16 E6/E7 genes, the lane number 1 is DNA size marker

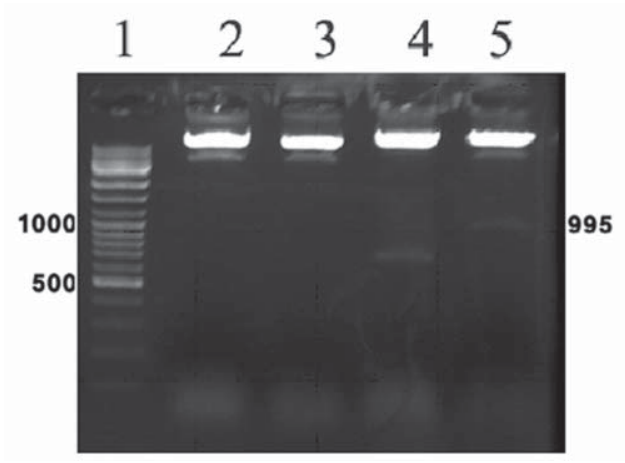

Fig. 2: Restriction enzyme analysis using different enzymes. The lane number 1 is DNA size marker. The lanes numbers 2 and 3 show single-digest plasmids using Hind III and Xba I enzymes, respectively. In the lane number 4, the fragment about 600 bp (E6 fragment) came out using Hind III and Bam HI and in the lane number 5 the fragment about 995 bp (E6 and E7) came out using the Hind III and Xba I enzymes.

RT-PCR (data are not shown) and Western blot analysis were used to characterize E6 and E7 proteins expression in E6-E7 DNA transfected cells using anti-E6 and anti-E7 monoclonal antibodies, respectively. Using anti-E6 and anti-E7 antibodies, the results showed that the protein band has approximately 17 kDa for E6 (Fig. 3) and about 11 kDa for E7 (data are not shown) in Western blotting.

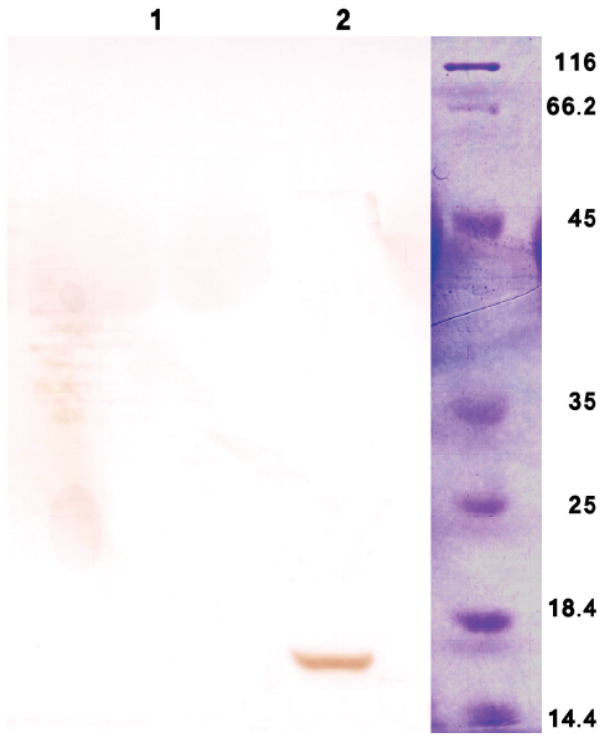

Fig. 3: Detection of E6 protein in transfected cells by Western blot analysis. CHO cells were transfected with the E6 expression construct. E6 protein was detected in lysates by western blot analysis using E6-specific monoclonal antibody. Western blot analysis showed a protein band with a size of approximately 17 kDa (lane 2) corresponding to E6 in pcDNA3/E6 transfected cells but not in pcDNA3 transfected cells.

Discussion

One of the major risk factors for the development of cancer in the anogenital tract especially uterine cervix malignant tumors is infection by some of the genital types of human papillomaviruses. The disease represents about 10% of cancer cases in women worldwide and the annual mortality rate (estimated for 1990) is in the order of 210000, therefore there is an urgent need for the efficient prevention and treatment (18). Prevention of infection by papillomaviruses especially the high-risk types is expected to significantly reduce the incidence of cervical cancer and other HPV-related malignancies. One of the important notes is the HPV E6 and E7 proteins are consistently expressed in tumor cells, and their expression is necessary and sufficient for cell transformation and maintenance of malignancy, hence, they are considered as the prime target antigens for prophylactic and therapeutic vaccines (19).

In this study, HPV 16 genotype was chosen because this genotype is commonly present in more than 50% of cervical cancers and it is the most prevalent high risk type in Iranian women. Previously we constructed cloning vectors containing HPV16 E6 and E7 genes (Accession No. DQ323982 and DQ323401 in Gene Bank) from Iranian patients (15,16). In the present study, an expression vector containing HPV16 E6 and E7 genes was designed and its recombinant protein expression was detected by RT-PCR and Western blotting. It is important to note the type of the cells is very important for recombinant protein production in high level, continuously. Several studies have used the CHO cells and they had gotten high-level recombinant protein production (19-21). CHO cells have showed

several advantages for the expression of heterologous genes which are included: i) the amplified genes are integrated into the host chromosome and are stably maintained even in the absence of continued drug selection; ii) a variety of proteins can be properly expressed at high levels in CHO cells; iii). CHO cells adapt well to growth in the absence of serum and can grow either attached or in suspension; and, iv) CHO cells can be scaled to greater than 5000 liters (21). In our study, we used the CHO cells for transfection and the genes were expressed in a reasonable level. Our protein production showed the same results in compared with the above studies.

In the other studies, one of the HPV oncoproteins, E6 or E7, was used alone or in combination with the other cellular or viral proteins for designing and construction the DNA vaccine (12,22-24). It is important to consider the cellular p53 is targeted for proteolysis by high-risk HPV E6 proteins. The high-risk HPV E7 proteins, when expressed in the absence of E6, results in increasing levels of p53 in either G-mediated cell cycle arrest or apoptosis, depending on the cell type. The proposed model involves the degradation of pRb by E7, resulting in the induction of E2F-1, which in turn activates p19arf, and interfere with the ability of mdm2 to regulate p53 stability. Thus E7 creates a signal that increases the p53 levels. E6, by promoting the degradation of p53, results in a lowering the steady-state level in the cell, counters this activity of E7. It seems the co-administration of E6 and E7 in a DNA vaccine results in increasing the stability of transfected cells and prevents from the apoptosis; thus it can increased the efficacy of the vaccine. Based on the other investigator results, we used both E6 and E7 genes in one desired DNA vaccine.

In this study, although HPV16 E6/E7 DNA vaccine was constructed, there are many questions which needed to be solved: Is the constructed DNA vaccine able to induce the immune responses in animal model? Is its efficiency more than the E6 or E7 DNA vaccine alone? How are the E6 and E7 genes, effect on each other in the vaccination using the desired vaccine and how strong is their immune responses? Has the DNA vaccine the same effects like the integrated HPV genome into the cell DNA in a natural infection? Also, it is important to know the immune responses level in co-administration of a DNA vaccine containing either E6 or E7 gene alone rather than a DNA vaccine including the both genes. In a DNA vaccine, what is the effect of the designed E7 location (up-stream or down-stream the E6 gene into the desired clone) on the gene expression and stimulation of the specific immune responses? To answer these questions, and other probable questions, we designed the DNA vaccine containing both E6 and E7 genes that it will be help us to understand complicated problems in HPV therapeutic DNA vaccines in future studies on the animal models.

Conclusion

In this study, the E6 and E7 genes were propagated from Iranian patient specimens and they used for designing and construction an expression vector. The designed vector can consider as a based for construction a therapeutic vaccine in suitable vectors using gene therapy protocols and after examination in animal model, it can be used in Iranian patients with cervical cancer.

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