Determination of human papillomavirus type 16 genotype and construction of cloning vector pTZ57R encoding HPV16 E7 gene.

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ABSTRACT

Objective: To isolate and construct a cloning vector containing the human papillomavirus (HPV)16-E7 gene as a target for application as a DNA vaccine.

Methods: The study was performed in 2005 in Iran. The E7 gene, one of the most important HPV oncoproteins and a target molecule for therapeutic vaccines, was amplified by polymerase chain reaction (PCR). The PCR product was cloned into a suitable cloning vector and confirmed by colony-PCR, restriction enzyme analysis, and sequenced.

Results: The desired plasmid was sequenced and indicated 99% homology with those mentioned in the Genbank.

Conclusion: The Iranian HPV16 E7 gene sequence is very similar to other sequences in the Genbank, and it can be used as a candidate gene in a therapeutic vaccine for Iranian patients with cervical cancer.

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Human papillomaviruses (HPVs) are DNA viruses with small circular genomes that cause epithelial hyperplasias ranging from benign papillomas (warts) to premalignant lesions that can progress to squamous cell carcinomas.[1,2] There are over 100 different HPV types, approximately 40 of which specifically infect anogenital tract mucosa.[3,4] These HPVs are classified as low risk, intermediate risk, and high risk, depending on the clinical prognosis of the lesions they cause.[5,6] High-risk HPVs, including HPV-16 and HPV-18, are associated with squamous intraepithelial lesions that can progress to cervical carcinomas.[7,8] High-risk HPV E7 oncoproteins target tumor suppressor protein (pRB) such as retinoblastoma,[9,10] at a higher efficiency than in low-risk HPV E7 proteins.[11] Interaction of E7 with hypophosphorylated pRB causes the disruption of growth-suppressive pRB-E2F complexes,[2,12] promoting G1-S cell cycle transition. Studying the high risk HPV early genes E6 and E7 has been of the central interest in related science and cancer studies. These genes are required for the maintenance of cellular transformation and constitutively expressed in the majority of precancer tumor cells. In this study, paraffin-embedded cervical cancer DNAs were extracted and identified for HPV positive samples by polymerase chain reaction (PCR). Human papillomavirus 16 (HPV16) genotyping was performed using specific primers and an HPV16 positive sample was used for E7 gene amplification. The purified E7 gene was cloned in pTZ57R/T vector (Fermentas) according to the manufacturer's instructions. To confirm HPV16-E7 open reading frame, it has been sequenced and published in GenBank (Accession No. DQ 323401). The aim of this study was isolation and construction of an expression vector containing the HPV 16-E7 gene as a target for DNA vaccine.

Methods. The study was performed in August 2005, in Tarbiat Modares University, Tehran, Iran, however, the samples were prepared from the archival tissue stored at the archival bank of the Cancer Research Institute (Imam Khomaini Hospital, Tehran, Iran). Five different blocks of genital specimens were collected from women with histological diagnosis of high grade intraepithelial lesions. The blocks were sectioned (20 μm) and subjected to DNA extraction.

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The DNA extraction from formalin-fixed and paraffin embedded tissues. Deparaffinisation was carried out by xylene/ethanol. Briefly, 1000 µl xylene was added to 20 µm sections, agitated, heated for 15 minutes at 37°C and then spun at 10500 g for 15 minutes. The supernatant was removed and the fresh xylene was added. These steps were repeated 2 times. Two identical washes with 100% ethanol for 30 minutes in 37°C were carried out and spun at 10500 g for 15 minutes. It was subjected to air drying before digestion.[13,14] Digestion was performed by adding, 150 µl of digestion buffer (Tris-Cl 100 mM pH=7.5, Tween 20 0.05% and proteinase K 3 µl of 10 mg/ml (Fermentas) to each tube and digested for 3 hours at 55°C, with gentle agitation several times. Proteinase K was inactivated at 92.5°C for 10 minutes.[13,14] Purification of the desired DNA was carried out by the phenol/chloroform method. After gentle agitation in 150 µl phenol/chloroform (1:1) mixture, samples were spun at 10500 g for 15 minutes. The upper phase was removed to another tube and this step repeated 2 times. After centrifugation, the upper phase was collected and added to 360 µl 100% ethanol: 0.03 M sodium acetate gently mixed and placed at -20°C for 2 hours. The precipitated nucleic acid was pelleted by centrifugation at 10500 g for 15 minutes, washed twice with 70% ethanol and air dried. The dried samples were resuspended in 30 µl sterile distilled water for further use.[15]

Polymerase chain reaction DNA amplification. The successful DNA extraction was assessed by PCR amplification of a 260bp fragment of β-globin gene using the published primers.[16] The GH20 and PCO4 (primers GH20 forward 5′- GAAGAGCCAAGGACAGGTAC -3′, PCO4 reverse 5′-CAACTTCATCCACGTTCACC -3′), 2 µl DNA sample, 1.5 mM MgCl2, 0.2 mM each dNTP, 5 pmol each primers and 1 u Taq polymerase (Cinagen, Iran). Amplification was carried out for 35 cycles (94°C for 30 seconds, 55°C for 45 seconds, 72°C for 45 seconds) after an initial denaturation step of 94°C for 5 minutes, on a Techne Thermal Cycler. The cycles were followed by a 5 minutes extension at 72°C, and the PCR product was visualized on a 1.5% agarose gel by ethidium bromide staining and UV photography. The L1 region of HPV genome was detected by PCR using the consensus primers MY09/MY11,[17] designed to amplify a segment of approximately 450 bp of the L1 gene of most genital HPV types. The PCR mixture consisted of 50 pmol each primers, 2 µl DNA sample, 1.5 mM MgCl2, 0.2 mM each dNTP, and 1u Taq polythermase (Cinagen, Iran). Amplification was carried out for 35 cycles such as β-globin gene PCR. The HPV16 genotype was determined by PCR using forward primer GTCAAAAGCCACTGTGTCCT and reverse primer CCATCCATTACATCCCGTAC. Each reaction was subjected to 30 amplification cycles using thermocycle-step parameters of 94°C for one minute, 56°C for 30 seconds, and 72°C for one minute, after an initial denaturation step of 94°C for 5 minutes. An additional 5 minutes was included at the final 72°C elongation cycle. The PCR reaction was prepared as described previously.[18] The HPV16 E7 ORF was obtained by PCR amplification using forward primer 5′- ACACGAGAATTCACCCAGCTGTAATCATGC - 3′, and reverse primer 5′- CCACGATCTGCCTCTAGAGATTATGGTTTCTGA - 3′. The forward and reverse primers contain EcoR I and Xba I sites. The resultant fragment was 342 bp. The amplification program was the denaturation step in 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 66.5°C for 45 seconds, 72°C for 45 seconds, and a final 72°C elongation step for 10 minutes.

Construction of the cloning vector containing the E7 gene. The resultant 342 bp HPV16E7 PCR product was inserted into the PTZ57R cloning vector. Colonies containing the plasmid were obtained by transformation of *Escherichia coli* (*E.coli)* DH5a bacteria as a host. The target colonies were selected by blue-white screening using IPTG, X-gal and then confirmed by colony-PCR, restriction enzyme analysis and sequencing.

Results. To check the quality of extracted DNA from tumor samples, β-globin gene PCR was performed for 5 samples. The results in **Figure 1** indicated that the β-globin was identified in 3-5 samples. **Figure 1** showed the 260 bp bands of the gene in sample 5, 6, and 7, while 2, and 3 were negative. To assess the presence of HPV in the β-globin positive samples, 3 positive samples were analyzed for HPV L1 PCR. The results in **Figure 2** showed that 2 of 3 were HPV L1 positive, and they showed approximately 450 bp fragments in PCR using MY09/MY11 consensus primers. **Figure 2** indicates that samples 4 and 5, showed a positive band while sample 2 was negative. To determine the HPV16 genotype positive samples, 2 HPV L1 positive samples were selected and the HPV16 genotype was determined by PCR using HPV16 specific primers. The results in **Figure 3** showed that one of the 2 samples was HPV16 positive. The HPV16 positive sample was selected and HPV16 E7 gene PCR was performed. The results obtain from HPV16 E7 PCR showed a 342 bp band which is shown in **Figure 4**. The HPV16 E7 fragment was prepared in large scale by PCR, and it was inserted into the cloning vector pTZ57R/T. The cloning vector was then inserted in *E.coli* DH5α bacteria and screened for positive colonies. The presence of desired plasmid
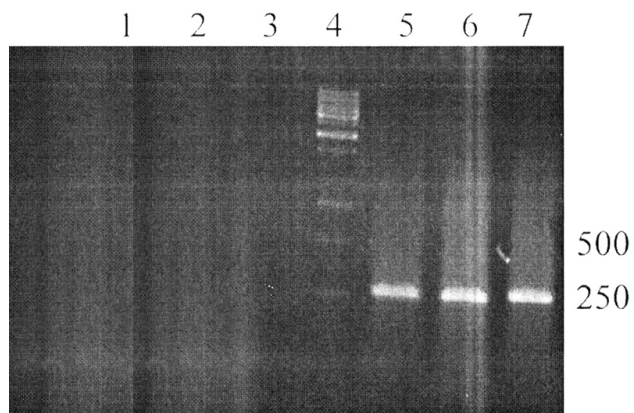
Figure 1 - Beta-globin (β-globin) gene polymerase chain reaction (PCR). Lane numbers 5, 6, and 7 showed a 260 bp fragment of β-globin gene. Lane 1 is the negative control of PCR and numbers 2 and 3 were the negative for β-globin gene. Lane 4 is the DNA size marker.
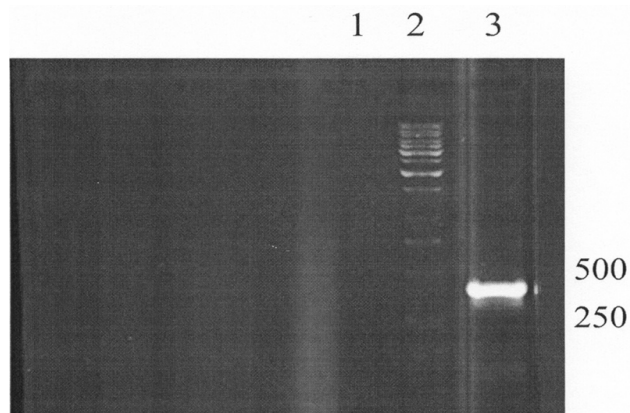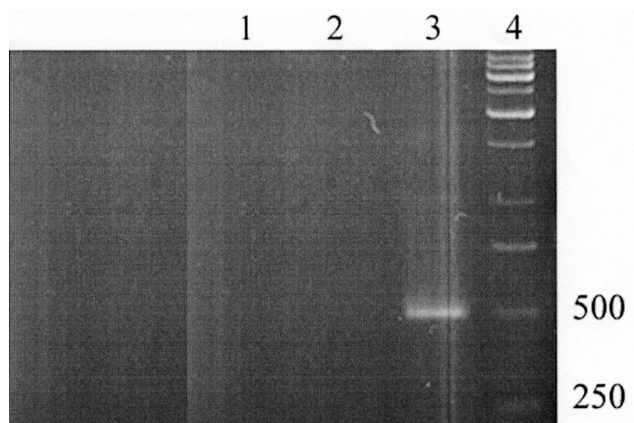

Figure 4 - The human papillomavirus (HPV) 16 E7 gene polymerase chain reaction (PCR). Lane number 3 showed a 342 bp fragment of HPV16 E7 gene. Lane number one is the negative control of PCR and Lane 2 is the DNA size marker.
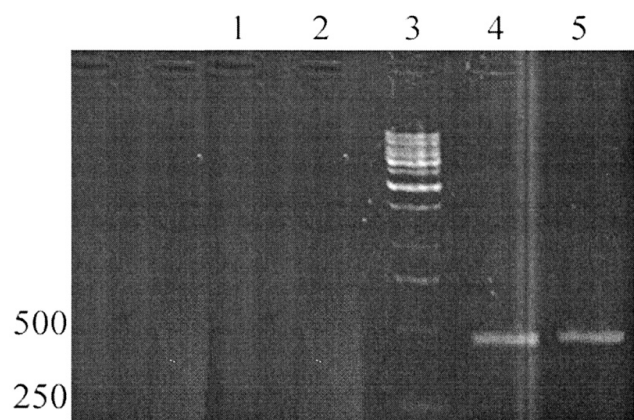

Figure 2 - The human papillomavirus (HPV) L1 gene polymerase chain reaction (PCR). Lane numbers 4 and 5 showed a 450 bp fragment of HPV L1 gene. Lane 1 is the negative control of PCR and lane 2 was the negative to HPV - L1 gene. Lane 3 is the DNA size marker.
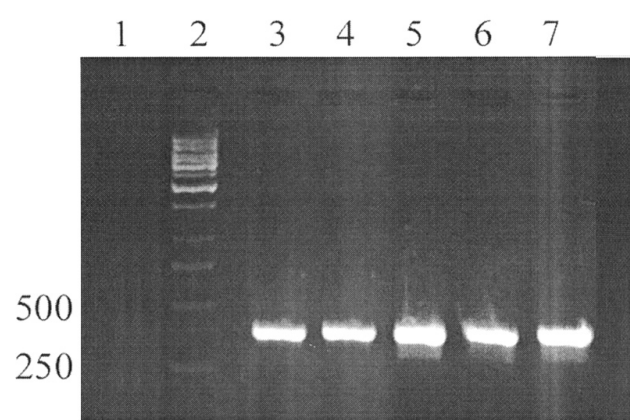
Figure 5 - The colony-polymerase chain reaction for colonies using E7 specific primers. Lane numbers 3, 4, 5, 6, and 7 showed a 342 bp fragment of human papillomavirus 16 E7 gene. Lane number one is the negative human papillomavirus 16 E7 gene and Lane 2 is the DNA size marker.

Figure 3 - The human papillomavirus (HPV) 16 specific region polymerase chain reaction (PCR). Lane number 3 showed a 500 bp fragment of HPV16 specific E6/E7 region. Lane 1 is the negative control of PCR and lane 2 was the negative to HPV - L1 gene. Lane 4 is the DNA size marker.
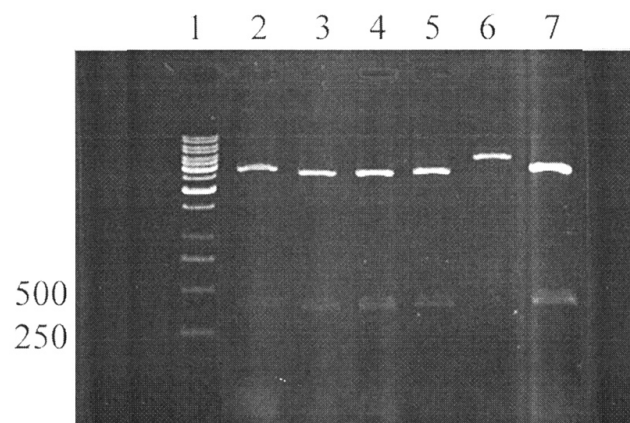
Figure 6 - Restriction enzyme analysis using Xba I. In lane numbers 2, 3, 4, 5, and 7, the fragment about 350 bp came out using the enzyme and they have right orientation and Lane 6 has left orientation and the fragment did not come out. Lane number 1 is the DNA size marker.

was confirmed by colony-PCR using E7 specific primers. As shown in **Figure 5**, the colonies containing desired plasmid were positive and their fragment was 342 bp. The colonies that were positive on colony-PCR were propagated and their plasmids were purified. We applied XbaI for exact conforming of the right orientation of inserted gene restriction enzyme analysis. The right orientation of constructed plasma, shows the fragment approximately 350 bp using XBA I (**Figure 6**), and it was purified and sequenced. Our sequence was confirmed by Genbank and indicated 99% homology with those mentioned in Genbank.

Discussion. Cervical carcinoma remains one of the most common malignancies world wide. Each year, approximately 500,000 cases are newly diagnosed with cervical cancer, with approximately 200,000 deaths annually.[19,20] Human papilloma virus type 16 (HPV16) is the predominant etiologic agent of cervical cancer and carries 3 transforming oncogenes E5, E6 and E7. Therefore, their products are unique antigens and can be ideally used as tumor vaccines.[19,21]

In the present study, DNA was extracted from paraffin-embedded cervical cancer specimens and screened for HPV 16 genotype since this genotype is commonly present in more than 50% of cervical cancers. The continued expression of the E7 ORF in malignant carcinoma makes E7 a potential vaccine candidate for HPV-associated cervical disease. In this study, we designed HPV16 E7 specific primers and the PCR product was cloned into the cloning vector. It was sequenced and reported as Iranian HPV16 E7 gene sequence and submitted in Genbank (accession No, DQ323401). The result obtained from sequencing of the cloned gene was compared with the published sequences in Genbank, and it showed that there was 99% homology between our product and those mentioned in Genbank. Compared to the E7 gene sequence from other countries, Iranian HPV16 E7 contains a high degree of similarity, and it can be used as a target gene in vaccine production. The HPV 16 E7 is currently a leading candidate for vaccine development against HPV causing genital infections. Immunization using the HPV 16 E7 DNA vaccine, as a novel vaccine technology, has shown promising effect for diseases arising from genital papillomaviruses, and it is a potent inducer of cellular and humoral immune responses.

References

1. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* 2004; 78: 11451-11460.

2. Streeck RE. A short introduction to papillomavirus biology. *Intervirology* 2002; 45: 287-289.

3. Sotlar K, Diemer D, Dethleffs A, Hack Y, Stubner A, Vollmer N, et al. Detection and typing of human papillomavirus by E6 nested multiplex PCR. *J Clin Microbiol* 2004; 42: 3176-3184.

4. Peh WL, Brandsma JL, Christensen ND, Cladel NM, Wu X, Doorbar J. The viral E4 protein is required for the completion of the cottontail rabbit papillomavirus productive cycle in vivo. *J Virol* 2004; 78: 2142-2151.

5. Roden RBS, Yutzy IVWH, Fallon R, Inglis S, Lowy DR, Schiller JT. Rapid communication: Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes. *Virology* 2000; 270: 254-257.

6. Jacobs MV, Snijders PJ, van den Brule AJ, Helmerhorst TJ, Meijer CJ, Walboomers JM. A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol* 1997; 35: 791-795.

7. Kawana K, Yoshikawa H, Taketani Y, Yoshiike K, Kanda T. Common neutralization epitope in minor capsid protein L2 of human papillomavirus types 16 and 6. *J Virol* 1999; 73: 6188-6190.

8. Thomas KK, Hughes JP, Kuypers JM, Kiviat NB, Lee SK, Adam DE, et al. Concurrent and sequential acquisition of different genital human papillomavirus types. *J Infect Dis* 2000; 182: 1097-1102.

9. Doorbar J. The papillomavirus life cycle. *J Clin Virol* 2005; 32 Suppl 1: S7-S15.

10. Meschede W, Zumbach K, Braspenning J, Scheffner M, Benitez-Bribiesca L, Luande J, et al. Antibodies against early proteins of human papillomaviruses as diagnostic markers for invasive cervical cancer. *J Clin Microbiol* 1998; 36: 475-480.

11. Heck DV, Yee CL, Howley PM, Munger K. Efficiency of binding to the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. *Proc Natl Acad Sci USA* 1992; 89: 4442-4446.

12. Chellappan S, Kraus VB, Kroger B, Munger K, Howley PM, Phelps WC, et al. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between the transcription factor E2F and the retinoblastoma gene product. *Proc Natl Acad Sci USA* 1992; 89: 4549-4553.

13. Cao W, Hashibe M, Rao JY. Comparison of methods for DNA extraction from paraffin-embedded tissues and buccal cells. *Cancer Detect Prev* 2003; 27: 397-404.

14. Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Res* 1999; 27: e12.

15. Sambrook J, Russell DW. Plasmids and their usefulness in molecular cloning. In: Molecular cloning. 3rd ed. New York (NY): Cold Spring Harbor; 2001. p. 31-38.

16. Resnick RM, Cornelissen MT, Wright DK, Eichinger GH, Fox HS, ter Schegget J, et al. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J Natl Cancer Inst* 1990; 82: 1477-1484.

17. Baay MF, Quint WG, Koudstaal J, Hollema H, Duk JM, Burger MP, et al. Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. *J Clin Microbiol* 1996; 34: 745-747.

18. Lanham S, Herbert A, Basarab A, Watt P. Detection of cervical infections in colposcopy clinic patients. *J Clin Microbiol* 2001; 39: 2946-2950.

19. Cope JU, Hildesheim A, Schiffman MH, Manos MM, Lorincz AT, Burk RD, et al. Comparison of the hybrid capture tube test and PCR for detection of human papillomavirus DNA in cervical specimens. *J Clin Microbiol* 1997; 35: 2262-2265.

20. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J Natl Cancer Inst* 1995; 87: 796-802.

21. Breitburd F, Kirnbauer R, Hubbert NL, Nonnenmacher B, Trin-Dinh-Desmarquet C, Orth G, et al. Immunization with virus-like particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J Virol* 1995; 69: 3959-3963.

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