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Isolation of Iranian Human *Papillomavirus* Type 16 E6 Gene and Construction of its Cloning Vector

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Abstract: Epidemiological and experimental studies have clearly shown that high-risk HPV infection is the main etiologic factor for cervical cancer. Recent studies have indicated that the E6 and E7 gene products play a critical role in cervical carcinogenesis. The E6 products interfere with the p53 functions and deregulate the cell cycle. The expression of E6, leading to the accumulation of DNA damage and the development of cervical cancer. We applied a polymerase chain reaction DNA amplification system using two distinct consensus oligonucleotide primer sets for the detection of human genital *papillomavirus* (HPV) sequences. The system incorporates one primer set designed to amplify a highly conserved L1 and a second primer set designed to amplify the E6 gene. This system was used to analyze fixed, paraffin-embedded tissue section in cervical carcinomas as well as normal cervical tissues, CaSki cells and other several control tissues for investigation of HPV DNA existence. The E6 PCR product was cloned into suitable cloning vector and confirmed by sequencing and restriction enzyme analysis.

Key words: Human *papillomavirus*, HPV16 genotype, E6 gene, gene cloning

INTRODUCTION

The primary cause of cervical cancer in women has recently been definitively linked to genital human *papillomavirus* (HPV) infections, which was detected in over 99% of cases worldwide (Streeck, 2002; Reeves *et al.*, 1994). The high-risk HPV types, including HPV type 16 (HPV-16) and 18 (HPV-18), are commonly associated with lesions that can progress to high-grade cervical intraepithelial neoplasia (Czegledy *et al.*, 1992; Hausen, 1991; Coutlee *et al.*, 1995). The incidences of cervical carcinoma show a highly global variation, ranging from 4.2 to 54.6/100,000 (Bosch *et al.*, 1995; Hausen, 1991). Cells constitutively expressing E6 and E7 proteins of HPV can bypass normal cell cycle checkpoints resulting in the accumulation of genetic damage which could ultimately result in malignant progression (Lee and Laimins, 2004; Comerford *et al.*, 1991; Gissmann *et al.*, 2001).

The *papillomavirus* E6 proteins are composed of approximately 150 amino acids. E6 from HPV-16 is made up of 151 amino acids. The E6 proteins are present at very low levels in cells, making *in vivo* analysis of E6 very difficult. It is quite evident that E6 is a multifunctional protein that affects cell growth and

proliferation. The E6-mediated activities include cell immortalization, transformation, tumor formation and apoptosis. In addition, E6 modulates transcription and telomerase activity (Rapp and Chen, 1998).

In this study, we extracted DNA from paraffin-embedded cervical cancer tissue samples and identified the HPV positive samples by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) analysis. The genomic E6 sequence was amplified from confirmed HPV16. The final PCR product was cloned into pTZ57R/T. The constructed vector containing the HPV16 E6 Open Reading Frame (ORF) was confirmed by DNA sequencing and submitted in Gene Bank (Accession No. DQ323982). The reported sequence is the first high risk HPV E6 gene from Iranian isolate, which has shown about 100% homology with two submitted sequence in Gene bank and 99% with others.

MATERIALS AND METHODS

DNA preparation: Collected samples (Tehran-Iran, 2005) were sectioned for pathologic evaluation and all of them presented histological diagnosis of high grade intraepithelial lesions.

In order to extract target DNA from 20 µm section paraffin embedded tissue was placed into the tube containing 1 mL Xylene and incubated at 37°C for 15 min. The tubes gently mixed for 15 sec and centrifuged at 10500 rpm for 15 min. The supernatant was removed, fresh Xylene added and these steps repeated two times. One milliliter of pure ethanol was added and the mixture was again centrifuged at 10500 rpm for 15 min and then transferred into new sterile 1.5 mL tube and the DNA was precipitated with ethanol. After centrifugation at 10500 rpm for 15 min, the upper phase was discarded and the pellet was dried at room temperature before digestion.

DNA extraction and storage of extracted DNA were done in a designated area free from amplification products. The concentration of extracted DNA was determined by spectrophotometry at 260 nm.

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⁻¹ (Fermentase) to each tube and digested for 3 h at 55°C, with gentle agitation every hour. Proteinase K was inactivated at 92.5°C for 10 min.

Digested DNA was purified by phenol/chloroform and precipitated with ethanol and air dried. The samples were re-suspended in 30 µL sterile distilled water and kept in -20°C for further uses.

DNA amplification: In order to assess quality of extracted DNA, a 206 bp fragment of β-globin gene was amplified using primers GH20 forward 5'- GAAGA GCCAAGGACAGGTAC -3', PCO4 reverse 5'-CAACTT CATCCACGTTTACC -3'. The reaction mixture was contained 2 µL DNA sample, 1.5 mM MgCl₂, 0.2 mM each dNTP, 5 pmol each primers, 1 unit Taq polymerase (Cinagen, Iran). Amplification was carried out for 35 cycles (94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec) after an initial denaturation step of 94°C for 5 min, on a Techne Thermal Cycler. The cycles were followed by a 5 min extension at 72°C and the PCR product was analysed using 1.5% agarose gel by ethidium bromide staining and UV photography.

PCR was applied using the consensus primers MY09/MY11 for HPV L1 region and a amplified segment of approximately 450 bp of the L1 gene belong to genital HPV types. PCR mixture consisted of 50 pmol each primers, 2 µL DNA sample, 1.5 mM MgCl₂, 0.2 mM each dNTP and 1 unit Taq polymerase (Cinagen, Iran). Amplification was carried out for 35 cycles like β-globin gene PCR program.

Restriction fragment length polymorphism (RFLP) analysis: A 70 µL of the MY09/MY11 PCR product was used for restriction digestion. The enzymes for RFLP

analysis of L1 region include: *BamHI*, *Dde I*, *Hae III*, *Hinf I*, *Pst I*, *Rsa I* and *Sau III AI* with corresponding buffers (Roche). After incubation at 37°C for 3 h, digested products were electrophoretically resolved on 12% poly acryl amid gel (PAGE) and silver nitrate staining (Epsaro *et al.*, 1994).

The HPV16 E6 ORF amplification: The HPV16 E6 ORF was obtained by PCR amplification using forward primer 5'- TAATCGAAGCTTAAACTAAGGGCGT-3' and reverse primer 5'- CGAAAGGATCCATATATTC ATGCAA TGTAG-3'. The forward and reverse primers contained *Hind III* and *BamH I* sites (underlined) respectively. The amplification program was included: denaturation step of 94°C for 5 min, 35 cycle of 94°C for 30 sec, 63.5°C for 45 sec, 72°C for 45 sec and final 72°C elongation for 10 min. The amplified PCR product was analyzed using 1.5% agarose gel electrophoresis and purified with gel extraction kit (Bioneer, Korea)

Construction of cloning vector containing E6 gene: The resulting 600 bp HPV16E6 PCR product was excised purified and inserted into the PTZ57R cloning vector. The resultant was used to transformed Ecoli DH5α cells as host. The colonies containing target construct were selected by blue-white screening using IPTG, X-gal and then confirmed by colony-PCR, restriction enzyme analysis. The resulted construct was send for sequencing (SEQLAB, Germany).

RESULTS

Conformation the quality of the extracted DNA by Beta-globin gene PCR amplification: To check the quality of extracted DNA from tumor samples, beta-globin gene PCR was performed for all of them. The results in Fig. 1 indicated that the beta-globin was identified in two of four samples. Figure 1 showed the 260 bp bands of the gene in two samples (lanes 5 and 6), while two samples (lanes 3 and 4) were negative.

Determination of HPV DNA in extracted DNA: To assess the presence of HPV in the confirmed beta-globin positive samples, two positive samples were applied for HPV L1 PCR. The results in Fig. 2 showed that one of two was HPV L1 positive and it showed about 450 bp fragments in PCR using MY09/MY11 consensus primers. Figure 2 indicate that lane 2 showed a desired band while the lane 3 was negative.

Restriction fragment length polymorphism (RFLP) for MY09/MY11 PCR product analysis: RFLP analysis was performed using MY09/MY11 PCR product and showed

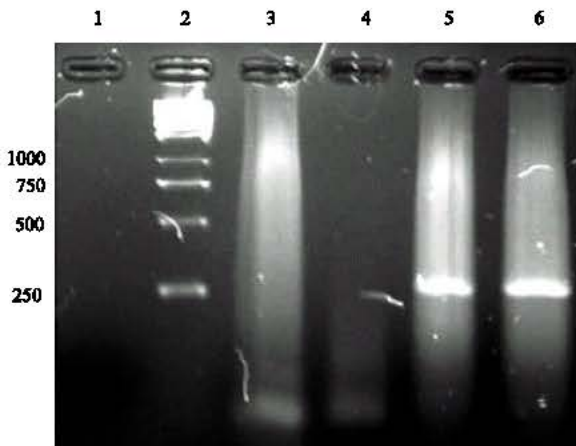


Fig. 1: Confirmation of the samples quality by Beta-globin gene PCR. The lane numbers 5 and 6 showed a 260 bp fragment of β -globin gene, the lane number 1 is the negative control of PCR and numbers 3 and 4 were negative. Lane 2 is DNA size marker

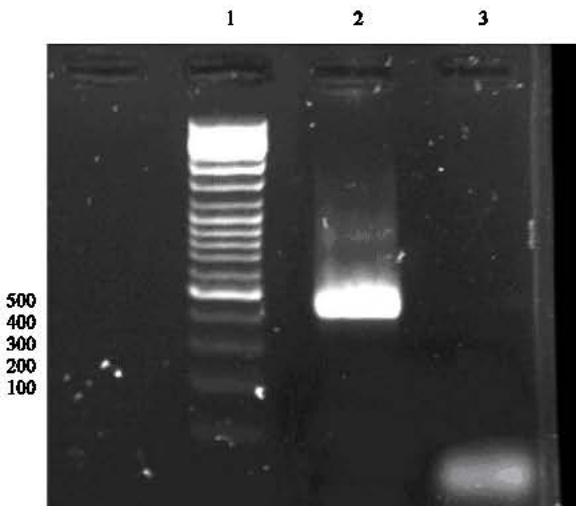


Fig. 2: HPV L1 gene PCR for confirmation of HPV positive samples. The lane numbers 2 showed a 450 bp fragment of HPV L1 gene, the lane number 3 was negative. Lane 1 is DNA size marker

the pattern of positive HPV16 in the HPV positive sample (Fig. 3) according to the published data by Epsaro *et al.* (1994).

HPV16 E6 gene PCR amplification: HPV16 positive sample was selected and HPV16 E6 gene PCR was performed. PCR of DNA samples for the E6 gene was positive and showed a 600 bp band in confirmed positive sample (lane 2 in Fig. 4) and CaSki cell line (lane 5 in Fig. 4).



Fig. 3: RFLP analysis on MY09/MY11 PCR product. It showed HPV16 genotype. Lane 1 is un-cut fragment and lane numbers 2 to 8 are restriction enzymes analysis by *Pst I*, *Sau III AI*, *Hae III*, *Rsa I*, *Hinf I*, *Bam HI* and *Dde I*, respectively, the lane number 9 is DNA size marker

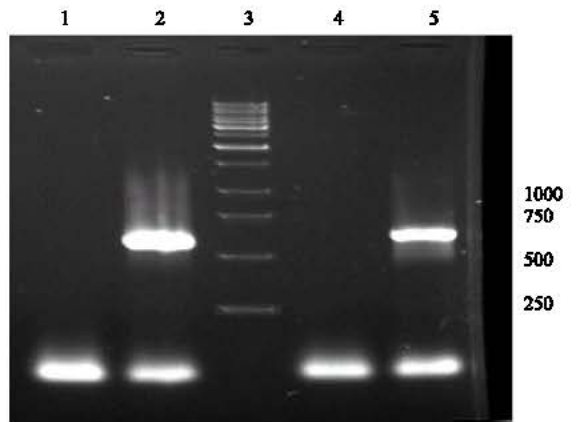


Fig. 4: HPV 16 E6 gene PCR. The lane number 2 (HPV-16 positive sample) and lane 5 (CaSki cells) showed a 600 bp fragment of HPV16 E6 gene, the lane number 1 and 4 are negative controls of PCR and Lane 3 is DNA size marker

HPV16 E6 gene insertion into the cloning vector: The HPV16 E6 fragment was prepared in large scale by PCR and it was inserted into the cloning vector PTZ57R (Fig. 5). The clone gene was transformed in *E. coli DH5 α* and screened for positive colonies. The white colonies containing desired gene was selected and confirmed by colony-PCR.

Confirmation of the desired plasmid by colony PCR: The presence of desired plasmid was confirmed by colony-PCR using E6 specific primers, as shown in Fig. 6.

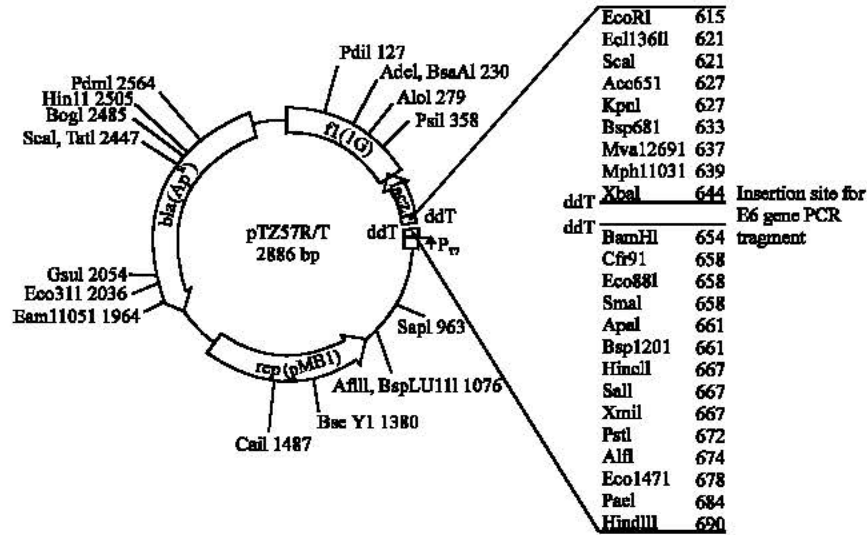


Fig. 5: A schematic diagram of the cloning vector and the insertion site for desired E6 PCR product

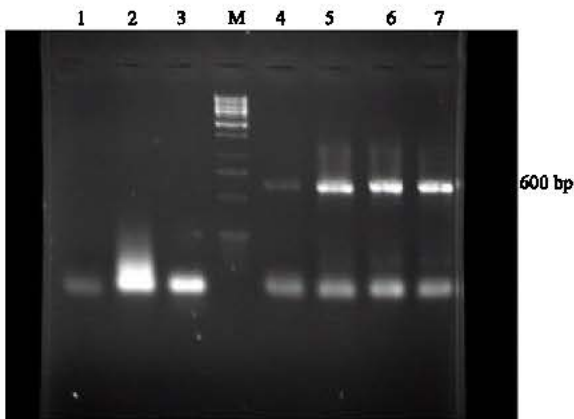


Fig. 6: Colony-PCR for colonies using E6 specific primers. The lane numbers 5, 6 and 7 showed a 600 bp fragment of HPV16 E6 gene, the lane numbers 2 and 3 are negative control (blue colony as tempelet). The lane number 1 is PCR negative control and Lane M is DNA size marker

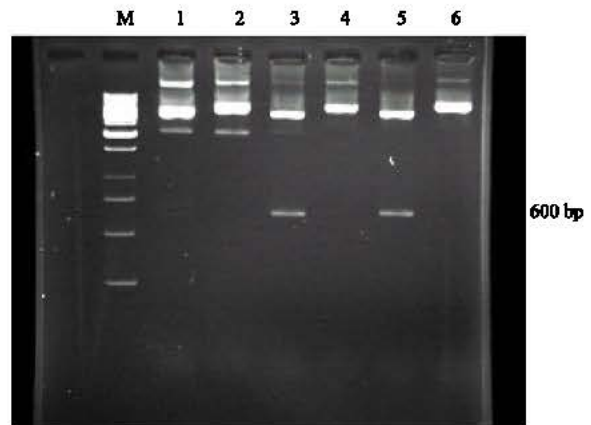


Fig. 7: Restriction enzyme analysis using Hind III and Bam HI. The lane numbers 3 and 5 show the fragment about 600 bp came out using the Hind III restriction enzyme. The lane numbers 4 and 6 show cutting pattern using Bam HI. The lane numbers 1 and 2 are un-cut plasmid, which has left orientation. The lane number 1 is DNA size marker

Identification of constructed plasmid by RFLP: The colonies containing target gene were cultured overnight and subjected to purification after mini preparation. In order to confirmed right orientation of cloned gene, it was digested with *Hind III* and a 600 bp fragment was appeared (Fig. 7).

The constructed plasmid was purified and sequenced by SEQLAB Company. The Iranian HPV16 E6 sequence was deposited in gene bank data base with accession number (DQ323982).

DISCUSSION

Human *papillomaviruses* (HPVs) are small double-stranded DNA viruses that induce hyper-proliferative lesions in epithelial tissues. A subset of HPV types infect epithelia in the anogenital region and are the etiological agents of cervical cancers. These HPV types are called high-risk and include HPV16, HPV18, HPV31 and HPV54 (Coutlee *et al.*, 1995). The oncogenic potential of these high-risk types is dependent on the cooperative action of

the two early viral gene products, E6 and E7, which bind and alter the activity of cell cycle-regulatory proteins. Selective retention and expression of these two viral oncoproteins is essential for HPV-induced oncogenesis (Ting and Manos, 1990; Comerford *et al.*, 1991). Sequence conservation usually is regarded as an indicator of functional importance (Ting and Manos, 1990).

In the present study, HPV 16 genotype was chosen for its commonly existence in more than 50% of cervical cancers. The continued expression of the E6 ORF in malignant carcinoma makes E6 a potential vaccine candidate for HPV-associated cervical disease. In this study, we designed HPV16 E6 specific primers and the PCR product was cloned into the cloning vector. It was sequenced and reported as Iranian HPV16 E6 gene sequence and submitted in Gene bank (accession No, DQ323982). The result obtained from sequencing of the cloned gene was compared with the sequences in Gene bank and it showed that there was more than 99% homology between our product and those mentioned in Gene bank. Therefore our data, like other investigations showed no significant differences between Iranian HPV16 E6 gene sequences and E6 gene sequence from other countries. In order to its homology with other isolate, our product can be used in future studies. Over the region sequenced, the inserted DNA HPV16E6 fragment was 99% homologous with those published sequenced in Gene bank and showed 100% homology with some limited sequences in Genbank such as AF 536179.1 and HPU89348. To study the preventive effect of E6, in activating immune systems against HPV infections, it seems that it is necessary to prepare pure recombinant E6 DNA or protein by expressing E6 gene in a proper host to make it available for future studies. Higher degrees of similarity make it attractive target sequence for antiviral and antitumor therapy against all emerged HPV16 isolates worldwide.

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