Isolation and cloning of human papillomavirus 16 L1 gene from Iranian isolate

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ABSTRACT

الأهداف: من أجل عزل واستنساخ وإظهار العوامل المحتوية على الجين HPV16–L1 كهدف لتطبيقه كتطعيم اتحاد جيني.

الطريقة: أجريت هذه الدراسة في عام 2007م في جامعة تاربيات موداريس – إيران. تم تكبير أربع عينات جينية من الحامض النووي DNA بواسطة استعمال تمهيدات محددة لنوع الفيروس الورمي الحليمي البشري المبني على الجين HPV16–L1 والجين E6 والجين E7. تم استنساخ منتجات تفاعل سلسلة الخمائر الناقلة PCR إلى استنساخ مناسب وعوامل الظهور، وتم تأكيد ذلك بواسطة تحليل الإنزيم والتكرار.

النتائج: تم تكرار البلازميدات المرغوبة وأشارت إلى 99% من التناسق مع الطول الكامل لتكرار الجين L1 في بنك الجينات.

خاتمة: إن هذا التقرير هو الأول عن تكرار طول الجين L1 بالكامل من معزولات إيرانية للجين HPV16–L1 في عينات نسيج السرطان العنقي المثبتة. أظهرت النتائج وجود 99% من التماثل بين منتجنا وتلك المنتجات المذكورة في بنك الجينات. في هذه الأيام يتم تركيب المحافظ الحموية الفارغة المسماة "جسيمات مثيل الفيروس"، مع استعمال أنظمة إظهار الميكروبات أو الخلويات. لذلك يمكننا أن نختم بأن تكرار طول الجين HPV16–L1، L1 الكامل من المعزولات الإيرانية مشابه جداً لتلك التكرارات الموجودة في بنك الجينات ويمكن استعماله كجين مرشح في التحصين الوقائي للسرطان العنقي.

Objective: To isolate and construct cloning and expression vectors containing human papillomavirus (HPV)16-L1 gene as target for application as recombinant vaccine.

Methods: The study was performed in 2007 in Tarbiat Modares University, Tehran, Iran. Four genital specimens DNAs were amplified with the use of HPV type-specific primers based on HPV-16 L1, E6, and E7 genes. The polymerase chain reaction products were cloned into suitable cloning and expression vectors and were confirmed by restriction enzyme analysis and sequencing.

Results: The desired plasmids were sequenced and indicated 99% homology with those submitted full length L1 sequences in the GenBank.

Conclusion: The results showed that there was 99% homology between our product and those mentioned in the GenBank. Nowadays empty viral capsids, termed "virus-like particles," are synthesized with the use of microbial or cellular expression systems. Therefore, it can be concluded that the Iranian HPV16 full length L1 sequence is very similar to the other sequences in the GenBank and it can be used as a candidate gene in prophylactic vaccine for cervical cancer.

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Human papillomaviruses (HPVs) are the predominant etiologic cause of epithelial hyperplasia ranging from benign papillomas (warts) to pre-malignant lesions that can progress to squamous cell carcinomas.[1,2] More than 120 different HPV types have been catalogued so far. More than 40 of these 120 different HPV types infect the epithelial lining of the anogenital tract and other mucosal areas of the body.[3] Human papillomavirus type 16 is the most prevalent genital HPV type and accounts for 50% of cervical cancer cases worldwide.[4] Approximately 80% of cervical cancer occurs in developing countries.[5]

The papillomavirus virion shells consist of L1 protein and L2 protein. The L1 protein alone or with L2 protein can self-assemble into virus-like particles (VLPs) when expressed in eukaryotic or prokaryotic expression systems.[6,7] Vaccines in clinical development are based upon VLPs, formed by heterologous expression

of the major capsid protein L1. Prophylactic HPV vaccination represents a promising strategy to prevent the occurrence of cervical cancer, and other HPV-related diseases. Administration of quadrivalent HPV (types 6, 11, 16, 18) L1 VLP vaccine (GARDASIL®, Merck & Co., Inc.) to 16–26-years-old induces potent anti-HPV 6, 11, 16, and 18 responses.[8] The vaccine has an excellent safety profile, is highly immunogenic, and has conferred complete type-specific protection against persistent infection and associated lesions in fully vaccinated women.[9]

In this study paraffin-embedded cervical cancer DNAs were extracted and identified for HPV positive samples by polymerase chain reaction (PCR). Human papillomavirus 16 genotyping was performed using a specific primer and a HPV16 positive sample was used for L1 ORF amplification and was cloned into pTZ57R/T and pET-28a vectors using the procedure described by the supplier (fermentas) after purification. In order to confirm HPV16- L1 open reading frame it has been sequenced and compared with submitted sequences in GenBank.

Methods. The study was performed in 2007 in Tarbiat Modares University, Tehran, Iran. The samples obtained with ethical approval from archival specimens of human paraffin embedded tissue. As paraffin embedded material is a main source for gene isolation, DNA was extracted from 8µm sections by deparaffination and digestion with proteinase K, deproteinised with phenol and chloroform and DNA precipitated with ethanol, as described by Wright and Manos.[10] This sample was confirmed for the presence of high grade neoplasia by histological examination. Deoxyribonucleic acid extraction was assessed by PCR amplification of a 260bp fragment of β-globin gene using the GH20 and PCO4 (primers GH20 forward 5′-GAA GAG CCA AGG ACA GGT AC-3′, PCO4 reverse 5′-CAA CTT CAT CCA CGT TCA CC-3′), 2 µl DNA sample, 1.5 mM Magnesium chloride (MgCl), 0.2 mM each Deoxyribonucleotide triphosphate (dNTP), 5 pmol each primer, one 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. Consensus region primers MY9 and MY11 are located within the L1 region of the HPV genome (**Table 1**). They are highly degenerated and thus consists 24 pairs of primers.[11,12] As a control for DNA extraction, mentioned β-globin primers were used. Conventional type specific primers (forward primer 5′-GTC AAA AGC CAC TGT GTC CT-3′; Reverse primer 5′-CCA TCC ATT ACA TCC CGA C-3′) were used for HPV-16 and produced a 499-bp product. For the HPV-16 PCR, the reaction mixture consisted of 2 mM MgCl, PCR buffer 10X, and dNTPs 200µM (Fermentas), primer at one mM, and 2 U Taq DNA polymerase (Fermentas). Two microliters of extracted DNA were used in a total PCR volume 25 µl. The primers with restriction enzyme site (forward primer with Sal1, 5′-GTT CCA GGG TCG ACA CAA TAT AC-3′; Reverse primer with Xho1, 5′-TTT GCC TCG AGC AGT TGT AGA GG-3′, producing a 1615-bp product) used for isolation of L1 ORF and cloning into expression vector. Appropriate negative and positive controls were included in each assay. Polymerase chain reaction products were analyzed by agarose gel electrophoresis and followed by ethidium bromide staining.

The *Escherichia coli (E. coli)* $DH5_a$ was used as a host during the cloning experiments and for propagation of plasmids. The cloning and expression vectors pTZ57R/T (fermentas) and pET28a (Novagen), were used for cloning and expression of full length of L1 ORF respectively. The PCR-amplified fragment was purified with a cleanup kit (fermentas) and then was cloned into pTZ57R/T vector using the overhang strategies described by the supplier (fermentas). Also, PCR products were cloned into multiple cloning site (MCS)of pET28a expression vector using Sal1 and Xho1 restriction enzyme sites at the end of the primers. To generate the pET-28a- HPV-16 L1 plasmid, the plasmid pET-28a and amplified L1 fragment were digested with Sal1 and Xho1. The resultant L1 fragment was ligated into the MCS of vectors. The ligated products were then transformed in *E. coli* $DH5_a$. The *E. coli* containing pTZ57R/T vector

Table 1 - Primers and amplicon size of MY9/11.

Primer	Sequences (5'-3')	Size (bp)	References
MY9	CGTCCMARRGGAWACTGATC	Approximately 450 for HPV 16	11
MY11	GCMCAGGGWCTATAAYAATGG		

Variable nucleotide positions are in bold and mean M = A or C, R = A or G, W = A or T, Y = C or T.
HPV - human papillomavirus

transformants were selected on LB-Amp (50 μm ml^{-1}) plate supplemented with IPTG and X-Gal whereas the *E. coli* containing pET-28a vector transformants were selected on LB-Kan (30 μm ml^{-1}). The target colonies were selected by blue-white screening using the IPTG, X-Gal and then were confirmed using the restriction enzyme analysis and were then subjected to Sanger method on an automated DNA sequencer (Seq. LAB Co., Germany) using universal primer against pET-28a. Analysis of sequencing was accomplished by chromas software (version 1.45-Australia) and showed on more than 99% identity with sequences submitted to GenBank.

Results. To check the quality of extracted DNA from tumor samples, β-globin gene PCR was performed (data not shown). The results in **Figure 1** indicated the positive sample for HPV-16 by PCR was positive for isolation of L1 ORF. The PCR products for HPV and HPV-16 were evaluated through electrophoresis on 1/5% agarose gel (**Figure 1**). The HPV-16 L1 ORF, encoding of major capsid protein, digested with Sal1 and Xho1 then was cloned into a multiple cloning site of pET-28a (**Figure 2**). The construct of pET-28a containing Iranian isolate of HPV16-L1 ORF was confirmed by restriction analysis using Sal1 and Xho1 (**Figure 3**) and applied for sequencing. Also, the construct pTZ57R/T-L1 was confirmed by restriction enzyme analysis using Sal1 and Xho1 (**Figure 4**).

Discussion. Infections by HPV-16 is causally associated with cervical cancer, one of the most common cancers worldwide.[13,14] Since their medical importance,

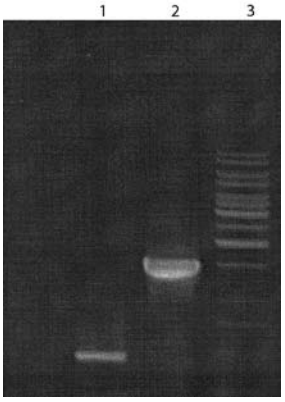

Figure 1 - Gel electrophoresis of polymerase chain reaction product, from left to right: Lane number one showed a 499-bp fragment for confirming of human papillomavirus (HPV)-16. Lane number 2 showed a 1615-bp ORF L1 HPV-16. Lane number 3 is the DNA size marker one kbp. The control has not been shown.

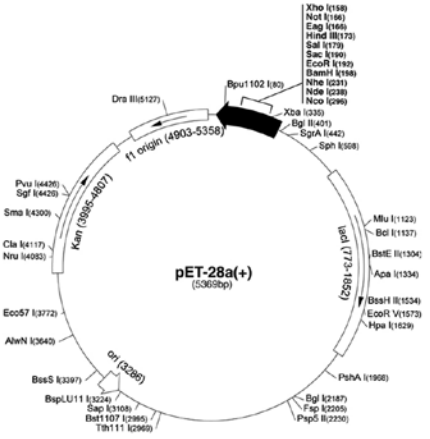

Figure 2 - The map of pET-28a as an expression vector (Novagen: Cat. No. 69337-3), L1 gene was cloned into a multiple cloning site between Sal1 and Xho1 restriction enzyme site of vector.

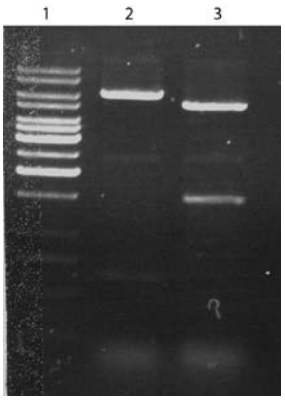

Figure 3 - Restriction enzyme analysis of recombinant plasmid pET-28a-L1. Lane one is the DNA size marker one kbp. Lane 2 is undigested recombinant plasmid. Lane 3 digested recombinant plasmid pET-28a-L1 with Sal1 and Xho1.

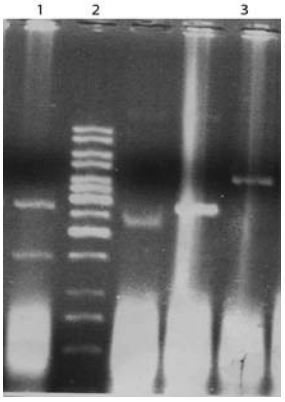

Figure 4 - Restriction enzyme analysis of pTZ57R/T-L1 construct. Lane one is Sal1 and Xho1 digested recombinant plasmid pTZ57R/T-L1. Lane 2 is DNA size marker one kbp. Lane 3 is recombinant plasmid pTZ57R/T-L1 undigested plasmid.

the HPVs have been most extensively studied. Initial infection requires access of infectious particles to cells in the basal layer, which for some HPV types is thought to require a break in the stratified epithelium. Studies have shown that DNA of HPV-16 can be readily amplified by PCR from epithelial cell of the cervix.[15] The papillomaviruse capsid consists of 2 structural proteins. The major capsid protein (L1) has a molecular weight of approximately 55 kd and represents approximately 80% of the total viral protein, which is expressed after L2 allowing the assembly of infectious particles in the upper layers of the epithelium.[1,16] Virus-like particles can be produced from different HPV by expressing L1 alone or the combination of L1 and L2 using mammalian or non-mammalian expression systems.[1] In 3 phase clinical studies, HPV-16 L1 VLP vaccines were observed to be generally well-tolerated and to induce more anti-HPV-16 responses.[17] At present, administration of quadrivalent HPV (types 6, 11, 16, 18) L1 VLP vaccine (GARDASIL®, Merck & Co., Inc.) to 16–26-years-old induces potent anti-HPV 6, 11, 16, and 18 responses.[8] In early studies, the HPV-16 L1 VLP vaccines was generally well tolerated and generated high levels of antibodies against HPV-16, so in the present study, DNA was extracted from paraffin-embedded cervical cancer specimens and screened for HPV 16 genotype and this genotype is commonly present in more than 50% of cervical cancers. The focus of this study was to investigate the possibility of cloning of HPV16-L1 ORF in prokaryotic expression system and in the future production, purification, and bioactivity of this product will be investigated.

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