Transient replication of human papillomavirus type 16 in HEK293T cells.

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Abstract

Our knowledge regarding human papillomaviruses (HPVs) replication remains very limited, in part due to the lack of a cell culture system for HPV replication. Here we present evidence that the plasmid containing the full-length HPV-16 genome can replicate and produce virus-like particles (VLPs) in HEK293T cells. At 48 h after transfection of the plasmid containing HPV-16 genome into HEK293T cell line, HPV-16 VLPs were observed from the cells with an electron micrograph, the virus DNAs existed in HEK293 cells for at least 60 h, and the fresh HPV-16 DNAs were synthesized at 20 h after transfection. The transcripts of E1, L1 and E1^E4 also were detected by RT-PCR. Therefore, HPV-16 genes expressed and HPV-16 VLPs formed in the cells. These results suggest that HEK293T cells allow HPV-16 replicates transiently.

Introduction

Trauma Human papillomaviruses (HPVs) are small dsDNA tumor viruses that are the etiologic agents of most cervical cancers and are associated with oropharyngeal cancers and benign lesions such as genital warts. The HPV's life cycle is tightly linked with the differentiation program of keratinocytes, moreover, the production of infectious HPVs is restricted to the terminally differentiated keratinocytes [1,2]. The initial cells infected by HPVs are thought to be basal epithelial cells of stratified epithelia, and the virus DNAs maintain at approximately 20 to 100 copies as extra chromosomal plasmids per infected basal cell; Following viral genome replication and cell division, one of the daughter cells migrates away from the basal layer and starts a program of differentiation, then high-level HPVs' later gene expression and virus assembly occur in the terminally differentiated suprabasal cells [3,4]. HPVs' replication assays suggest that both viral proteins E1, a DNA helicase, and E2, a transcriptional activator are essential for HPV DNA replication [5,6], and E1^E4 may affect the replication of viral genomes [7,8]. However, the evidence suggests that E1 and E2 are not entirely essential for stable replication of papillomaviruses [9]. The HPVs late (L) ORFs encode the major capsid protein L1 and the minor capsid protein L2, and L1 and L2 are expressed during the late phase of virus life cycle only in the most differentiated epithelial cells, then the HPVs L1 and L1-L2 can auto-assemble to form virus-like particles (VLPs) [10,11].

The studies on the viral DNA replication and the life cycle of HPVs had hampered by the limited availability of appropriate cell culture systems. Initial studies on HPVs life cycle involved descriptive studies of the distribution of viral genomes and gene products in naturally occurring lesions in humans. In 1984, the organotypic raft culture system was developed, and then it had been proven that the HPV genome could replicate and the HPV virions were produced in the system [12,13]. In 1996, circular HPV-31genome was cotransfected with a neomycin resistance gene into human foreskin keratinocytes (HFKs), then transfected cells were subjected to G418 selection, and the stable cell line maintaining HPV31 genome was generated [14], and these models have been used as *in vitro* experimental systems for studying HPVs reproduction and pathogenesis [15,16]. In recent years, considerable progress has been made in studying the HPV life cycle by using various tissue culture models, an improved method for efficient production and passaging of HPV has been described [17], the U2O S cell system has been used as an efficient tool for studying HPV DNA replication [18-21], and a new system consists of a novel HPV replicon and an organotypic raft culture have been developed, the HPV-DNA is maintained stably in normal human keratinocytes for a long period and the viral vegetative replicate by the system [21].

The incidence of cervical cancer in Xinjiang, China, is high and closely associated with HPV-16 infection [22]. In this work, the full-length genome of HPV-16 was isolated from cervical cancer biopsies that had been surgically obtained from Uygur patient hospitalized in the Affiliated Hospital for Cancer in Xinjiang, China [23], then the HPV-16 genome was cloned into pCR®-XL-TOPO® vector and transfected into HEK293T cell line, we found that the virus VLPs are produced and HPV-16 DNAs replicate transiently in the HEK293T cell line,

so the described cell system can be used for studying HPVs replication.

Materials and Methods

Clinical specimens

Histologically confirmed cervical cancer biopsies that had been surgically obtained from Uygur patients hospitalized in the Affiliated Hospital for Cancer in Xinjiang, China. The tissue DNA was extracted from the specimens using a Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China).

Enzymes and chemicals

Restriction enzymes, nuclease Ba131, T4 DNA ligase, Taq DNA polymerase (including Ex Taq and LA Taq), genomic DNA extraction kit, plasmid purification kit and agarose Gel DNA fragment recovery kit were products of TaKaRa, Dalian, China. Nucleic acids hybridization kit was the product of Roche Diagnostics, Germany. The plasmid pCR-XL-TOPO was the product of Invitrogen, Carlsbad, CA. All enzymes, kits, and the plasmid were used according to the manufacturers' recommendations.

Plasmid construction

The full-length HPV-16 genome was amplified by LA-PCR (sense primer: 5'-ATAGGGCCCTTCTGATCCTTCTATAGTTTCTTTAGTG-3', antisense primer: 5'-ATAGGGCCCACAGGATCTACTGTTAAAG, the underlined sequences are Apa sites) from the tissue DNAs of the cervical cancer biopsy, and the primer pairs are located on both sides of HPV-16 L2 gene's unique Apa I site. The LA-PCR conditions were performed at 95°C for 5 min and then followed by 30 cycles at 94°C for the 30 s, 55°C for 30 s, and 68°C for 7 min. Then the HPV-16 genome was cloned into pCR-XL-TOPO (pTXJHPV-16), the recombinant plasmid DNA was digested with several restriction endonucleases and sequenced (by Sangon Biotech Co. Ltd, Shanghai, China). The plasmid pcDNA3-L1L2 carrying HPV-16 L1/L2 was constructed previously [24].

Cell culture and transfection

HEK293T cells were grown at 37°C in DMEM (Invitrogen USA) supplemented with 10% FCS and 100 µg/ml penicillin plus 100 U/ml streptomycin, in 5% CO_2 atmosphere. 2×10^6 Cells were transiently transfected with 4 µg of pTXJHPV-16, linearized pTXJHPV-16 (digested with Apa I), pcDNA3.1 (negative control) or pcDNA3.1-L1L2 (positive control) using Lipofectamine 2000 (Invitrogen USA) according to the manufacturer's instructions respectively.

Electron micrograph

After 48 h, the transiently transfected cells were harvested by scraping, then centrifuged at 3,000 r/min for 5 min and washed twice with ice-cold phosphate-buffered saline (PBS). The cells were resuspended in 2 ml of ice-cold PBS and lysed by sonication for 30 s, then centrifuged at 4°C for 10 min at 8,000 g. The supernatant fluid was collected for freeze-drying, then the freeze-drying products were dissolved into 50 µL ice-cold PBS, and stored at -80°C. Virions were observed by electron micrographs after negative staining with phosphotungstic acid.

DNA and RNA extraction

2 h, 20 h, and 40 h post-transfection, the total cellular DNAs were isolated by Hirt extraction [25] after washing to remove excess salt and the cellular DNAs were examined for the presence of HPV-16 DNA. 12 h post-transfection, the total cellular RNAs were isolated from 50-100 mg of transfected cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The total RNAs were examined by 1% agarose gel electrophoresis, the quantity was determined based on absorbance at 260 nm (A260), and the purity was analyzed based on the absorbance ratio at 260 and 280 nm (A260/280), then the cellular RNAs were examined for the presence of HPV-16 transcripts.

Detection of HPV-16 E1 and E2 by PCR

E1 and E2 were amplified by PCR from the cellular DNAs, E1 primers (5'-TTGTGTG CCCCATCTGTTCTC 3' and 5' TGGTCACGTAG GTCTGTACTATCATTT-3') and E2 primers (5'-TAAGTTTGCACGAGGACGAGG-3' and 5'-GCACGCCAGTAATG-TTGTGG-3') were synthesized by Sangon Biotech Co. Ltd, Shanghai, China. PCR conditions of E1 were performed at 95°C for 5 min and then followed by 30 cycles at 94°C for the 30 s, 56°C for 45 s and 72°C for the 90 s; these of E2 were performed at 95°C for 5 min and then followed by 30 cycles at 94°C for the 30 s, 55°C for 45 s, and 72°C for 60 s. Then the PCR products were electrophoresed through 1% agarose gels containing ethidium bromide.

Detection of HPV-16 transcripts by RT-PCR

HPV-16 transcripts were examined from the cellular RNA by RT-PCR. First, the cellular RNAs were treated with DNAse to remove contaminating DNA, then the cDNA was synthesized from 1 µg of the cellular RNAs using AMV reverse transcriptase XL (TaKaRa, Dalian, China), and the RT-PCRs were performed using One-step RT-PCR Kit (TaKaRa, Dalian, China). The E1 primers as previously described. E1^E4 primers (5'-ACAAGCAGAACCGGAC-3' and 5'-CTCTGATCTTGGTCG-CTG-3'), L1 primers (5'-ATTCCTATAGTTCCAGGGTCT CCA-3' and 5'-ACTTACAGCTTACGTTTTTTGCGTT-3'), β-actin primers (5'-AGCCATGTAC-GTAGCCATCC-3' and 5'-CTCTCAGCTGTGGTGGTGAA-3') were synthesized by Sangon Biotech Co. Ltd, Shanghai, China.

Results

## Restriction enzyme mapping and sequence analysis of pTXJHPV16

An about 8000-bp DNA fragment was obtained by the PCR. The DNA fragment was cloned into pCR-XL-TOPO, both of 7,963- and 3,457-bp DNA fragments were detected from the recombinant DNA digested with Apa I, both of 6,094 and 5,389 bp fragments were detected from the DNA digested with BamH I, and a unique 11,482-bp DNA fragment was detected from the DNA digested with Xho I (Figure 1), and then the recombinant plasmid was designated pTXJHPV16. Sequence analysis indicated that the cloned HPV-16 genome consists of 7963 bp (Gene Bank accession No. FJ006723.1), and there is 63 nucleotides (nt1311~1773) duplication [26] in the HPV-16 E1.
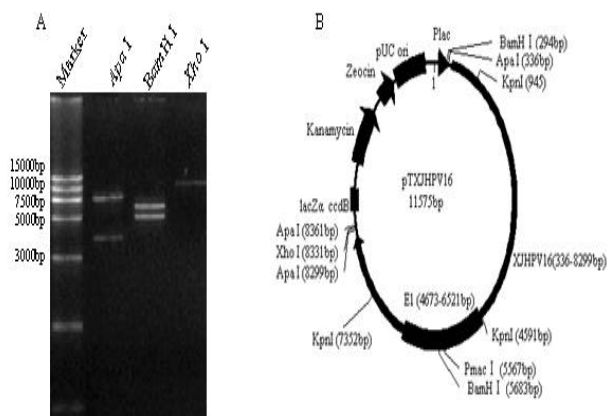
Figure 1. Restriction enzyme analysis of the plasmid and schematic representation of pTXJHPV16 structure. A: 1% agarose gel electrophoresis analysis the restriction enzyme digestion of pTXJHPV16; B: Schematic representation of pTXJHPV16 structure.

HPV-16 VLPs in HEK293T cells

The final step in the HPV life cycle is the assembly of viral particles. To investigate the possibility that the HEK293 cells allow for HPV-16 replication, the lysates of the transfected cells were prepared and examined by electron microscopy. Electron microscopic examination showed the presence of viral particles of the proper shape and approximately 50 nm in diameter from the cells transfected with pcDNA3.1-L1L2, pTXJHPV-16, and linear XJHPV-16 (Figure 2).
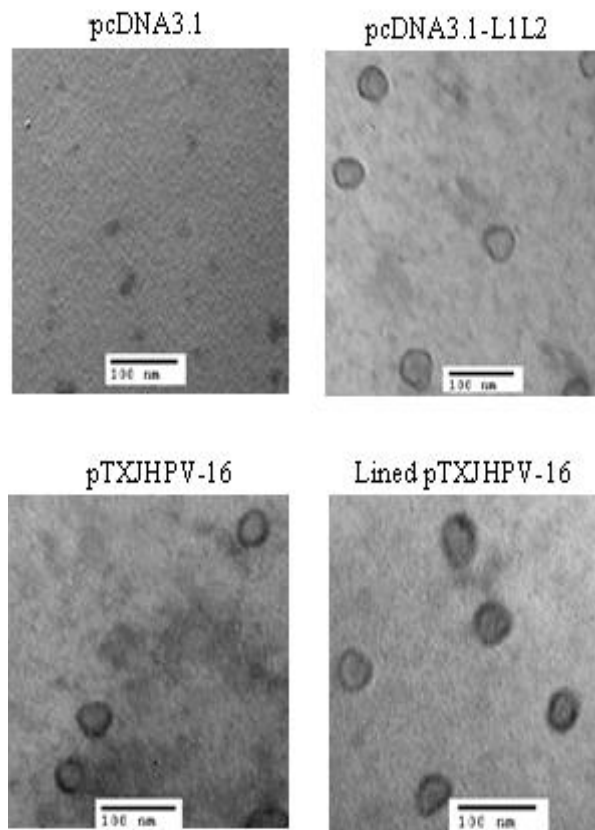
Figure 2. Electron micrographs of VLPs from the HEK293T cells transfected with pcDNA3.1 (negative control), pcDNA3.1-L1L2 (positive control), pTXJHPV-16, and linear pXJHPV-16. (Staining with phosphotungstic acid, 100,000×).

## Amplification of viral DNA in HEK293T cells

There are two existence states of HPV-16 in host cells: extrachromosomal form (episome) and integral form, and it is usually integrated into the chromosomes of malignant cells [27,28]. Integration usually disrupts the E1 and/or E2 genes, potentially leading to the deregulation of viral gene expression. In this works, the HPV DNA was harvested from the transfected cells and analyzed for the presence state by the previously described method [29,30], two overlapping fragments of the E1 (nucleotide 859-2866) and E2 (nucleotide 2721-3909) were detected by PCR (Figure 3), so HPV-16 E1 and E2 have not been disrupted and pTXJHPV16 existed as episome in the cells.
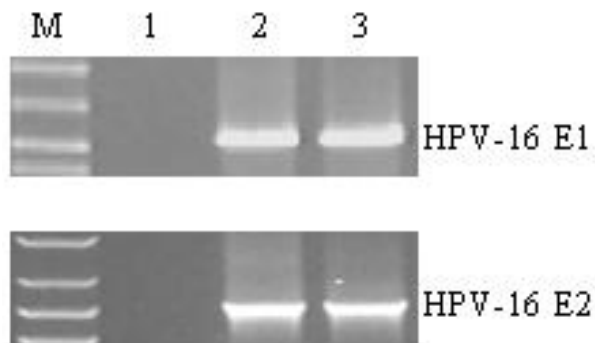
Figure 3. Analysis of PCR products for HPV-16 E1 and E2 by gel electrophoresis. Lane M: DNA marker; Lane 1: water (negative control); Lane 2: the total DNA from cells at 40 h posttransfection; Lane 3: pTXJHPV-16 (positive control).

The total DNAs were extracted from cells at 2 h, 20 h and 40 h post-transfection, respectively, and then hybridized with HPV-16 E1 digoxin-labeled probes. Digestion of the DNA with PstI yielded one fragment of 2.8 kb (Figure 4A). The decreases of HPV-16 DNAs were detected after transfection from 2 h to 20 h, while the quantity of HPV-16 DNAs began to increase by 40 h post-transfection (Figure 4A).

To detect the fresh HPV-16 DNAs produced in the cell, the total DNAs from cells at 2 h, 20 h, and 40 h post-transfection were digested with Dpn I, which can cleave the methylated DNAs but cannot cleave the unmethylated fresh DNAs, then hybridized with HPV-16 E1 digoxin-labeled probes. An 11.4 kb fragment was observed at 20 h post-transfection, while the fragment didn' t yield at 2 h and 40 h post-transfection (Figure 4B), so the fresh HPV-16 DNAs were synthesized at about 20 h post-transfection in the cells.
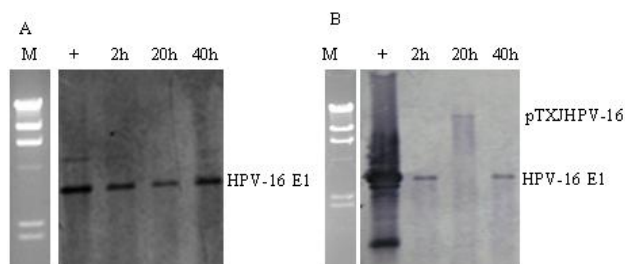

Figure 4. Replication of HPV-16 DNAs in HEK293T cell lines. The total DNAs from cells transfected with pTXJHPV-16 were digested with Pst I (A) or Dpn I (B), then the DNAs were transferred to positively charged nylon membranes and hybridized with HPV-16 E1 digoxin-labeled probes. Lane M: DNA marker; Lane +: pTXJHPV-16 (positive control); Lane 2 h, 20 h, and 40 h: the total DNAs from cells at 2 h, 20 h and 40 h post transfection.

The analysis of viral transcript in HEK293T cells

RT-PCR was performed to validate the transcription of the viral genes in HEK293T cells. The genes selected for analysis include early gene E1, late gene L1, E1^E4 transcript and β-

actin (as an internal control), and all genes' transcripts were detected (Figure 5).
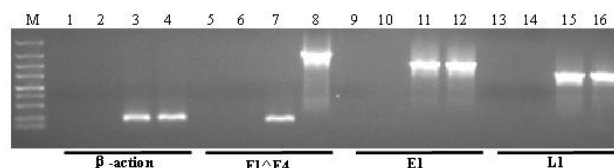
Figure 5. HPV-16 transcripts were detected by RT-PCR in the RNA isolated from the cells transfected with pTXJHPV-16. lane M, DNA Marker; lane 1, 5, 9, 13 water (negative control); lane 2, 6, 10, 14 the cellular RNA (control for eliminating residual pTXJHPV16 in the RNA); lane 3, 7, 11, 15 the cDNA of the cellular RNA; lane 4, 8, 12, 16 pTXJHPV16 (positive control).

Discussion

Studies on HPVs replication have been hampered by the inability of these viruses to replicate to high titer in the laboratory. At present, several suitable experimental systems for studying HPVs replication have been used, that include the xenografting tissues or cells of HPV-containing, athymic mice and the organotypic raft culture system of differentiated epithelial [2,3].

Much research has shown the HPV L1 express and self-assemble into VLPs in several systems [31]. including Mammalian cells, insect cells, yeast cells, plants, and bacteria. In this work, the recombination plasmid pXJHPV-16 carrying complete HPV-16 genome, while the L2 was disrupted at the gene's Apa I site. The results show that the L1 transcript was detected from the transfection cells and HPV-16 VLP of approximately 50 nm were found by transmission electron microscopy in the cells transfected with pTXJHPV16 or linear pTXJHPV16, and then the cells transfected with pTXJHPV16 were used for further research.

The high-risk HPVs are easier to be integrated into a host cell, and the integration usually disrupts the E1 and/or E2. For example, in the SiHa cell line, the HPV-16 genome is integrated into the cellular DNA with disruption and partial deletion of the viral E2 [30,32], so E2 can' t be detected from SiHa genomic DNA. Cricca used 6 primer pairs spanning the E1 or E2 to detect the disruption/deletion within E1 and E2 [29]. Here we designed two primer pairs spanning E1 (nucleotide 859-2866) and E2 (nucleotide 2721-3909), and both E1 and E2 were detected from the transfected cells. Therefore, pTXJHPV16 existed as an episome in the transfection cells.

The most highly expressed HPV proteins during productive infection are the E1^E4 proteins, which consists of the first five amino acids of E1 fused to E4 coding sequences. The E1^E4 proteins are synthesized in the late phase of the viral life cycle from spliced transcripts that initiate at the late promoter. In HPV' s differentiation-dependent life cycle, E1^E4 synthesis occurs concurrently with genome amplification [33,34] and E1^E4 protein plays a significant role in promoting HPV genome amplification and S phase

maintenance during differentiation [35], And just as mention in the introduction, HPV E1 plays an important role in HPV DNA replication. In this work, the transcript of E1^E4 and E1 have detected from the cells transfected pTXJHPV16 (Figure 5), and then the E1^E4 and E1 could promote the viral DNA's replication [36,37].

Conclusion

Following virus binding and entry, virions migrate to the nucleus and establish their genomes as a multi-copy extra chromosomal plasmid, which are maintained at approximately 20 to 100 copies per infected basal cell. The stable maintenance of HPV episomes requires persistent replication of viral DNA, the percentage of differentiated cells for amplifying viral DNAs are often very low. In our work, HPV-16 DNAs were decreasing in the cell transfected with pTXJHPV16 from 2 h to 20 h, which was caused by DNase digesting HPV-16 DNAs in the cells. However, the quantity of HPV-16 DNAs began to increase after 40 h, which suggests that HPV-16 DNAs ponderously replicate in HEK293T cells. At the same time, the unmethylated fresh HPV-16 DNAs were detected in the cells after transfected for 20 h but were not detected for 40 h, which affirm that HPV-16 DNAs transiently replicate in the cells. However, the quantity of HPV-16 DNAs has not increased in the cells after transfected for 20 h, this result may be due to a less refresh HPV-16 DNAs than the DNAs digested by DNase at the same time. In this study, we found that pTXJHPV-16 could be maintained at least for 60 h in culture, and then a lot of transfected cells died after 60 h.

In a word, we found that HPV-16 can replicate in HEK293T cells transiently and provided a new method for studying the replication mechanism of HPV-16.

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