

Lineage and phylogenetic analysis of HPV-16, -18 in saliva of HNSCC patients

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Aim: Head and Neck Squamous Cell Carcinoma (HNSCC) is a global health problem whose incidence varies by geographic region and race according to risk factors. Human papillomavirus (HPV) infection is a significant risk factor for HNSCC. HPV-16 and HPV-18 are two forms of HPV that are carcinogenic. HNSCCs that are HPV positive have a better prognosis rather than HPV negative. The purpose of this research was to characterize HPV-16, -18 variations in the saliva of HNSCC patients by examining the genetic diversity of HPV-16, -18 utilizing the full E6, E7, and L1 genes. **Methods:** The case-control research included 15 patients with HNSCC and 15 healthy volunteers. Unstimulated entire saliva samples were obtained from the case and control groups by spitting method. Genomic DNA was isolated from all saliva samples. A PCR reaction was used to determine the presence of HPV in saliva. HPV-positive samples were genotyped and data were analyzed. We conducted a variant study on the HPV-16, -18 E6, and E7 genes. **Results:** Three patients with HNSCC were HPV-positive for two HPV genotypes out of 30 people diagnosed with HPV-DNA. HPV-16 and -18 were the most common genotypes. The HPV-16, -18 E6, and E7 genes were sequenced and compared to the HPV-16, -18 (E6, E7) prototype sequence. In all, HPV-16 lineages A1 and HPV-18 lineages A3 were discovered. **Conclusion:** Regarding the variation of HPV found in Iranian HNSCC patients, the need for further studies in HPV genotyping was seen. Sequencing HPV genes in HNSCC may help answer questions about HPV genotyping in the Iranian population. HPV genotype analysis aids in the development of vaccinations against HNSCC, halting disease progression and preventing HPV-associated HNSCC.

Keywords: Squamous cell carcinoma of head and neck. Saliva. Alphapapillomavirus. Phylogeny.

Introduction

Squamous cell carcinoma (SCC) accounts for 90% of oral cavity cancers, and about 400000 new instances of head and neck squamous cell carcinoma (HNSCC) are diagnosed each year¹. Men are 1.5 times more likely than women to develop HNSCC². The prevalence of HNSCC varies by geographic region and race due to risk factor differences³. The incidence of HNSCC is higher in India, Pakistan, and Bangladesh in the southern part of Asia^{1,4}. It may appear on the mucosa as a red, white, or red-white prominence. It may also be portrayed as a severe wound with noticeable edges. It is often linked to lymph node swelling⁵. HNSCC is a complex illness with a variety of intrinsic and extrinsic risk factors that contribute to its development. Tobacco and alcohol usage, infections, and dietary variables are examples of extrinsic risk factors. Iron deficiency anemia and alterations in cell cycle control genes are among the inherent risk factors for HNSCC^{6,7}. In contrast to other viruses, human papillomavirus (HPV) has a significant role in HNSCC pathogenesis among extrinsic risk factors⁸. HPV is a DNA virus that attacks the skin and mucous membranes. There are about 30 different types of HPV viruses that have been linked to benign and malignant epithelial lesions⁹. Furthermore, high-risk HPV strains such as HPV-16 and HPV-18 are thought to be etiologic factors for HNSCC^{10,11}. The presence of HPV-16 in exfoliated oral epithelial cells may raise the risk of HNSCC and oropharyngeal cancer by 4 and 14 times, respectively¹². According to reports, HPV-16 is the most common kind in nearly all geographical areas of Iran, followed by HPV-18^{13,14}. HPV-16, HPV-18, HPV-11, and HPV-52 as the most frequently identified HPV types in Iranian patients with HNSCC^{12,15}. Although the data on HPV types in Iran is well-documented, there is little information on HPV variations in HNSCC. The current research sought to examine basic data of HPV-16 and HPV-18 variants in Iranian patients with HNSCC by examining the genetic variability of HPV-16 and HPV-18 utilizing the whole E6, E7, and L genes. The findings of our study might provide justification for future research into HPV genetic epidemiology, pathogenicity, and evolution.

Material and method

Ethical statement

This study was approved by the Tehran University of Medical Sciences Ethical Committee (Ethical code IR.TUMS.DENTISTRY.REC.1398.036). After describing the study objectives, written informed consent was obtained from all case and control group subjects.

Study population

This case-control study was performed in Imam Khomeini Hospital, Tehran University of Medical Sciences (Tehran, Iran). 15 HNSCC patients and 15 healthy volunteers as the control group enrolled in the study. HNSCC of all patients was confirmed with the histopathologic examination, and they had not undergone surgery, chemotherapy, and radiotherapy. The study participants were not affected by any

other malignancy or systemic disease. In the intraoral examination, there was no active dental and periodontal infection. The control group was matched with the case group for age, sex, tobacco, and alcohol consumption. Unstimulated whole saliva samples were collected from the case and control group using the spitting method with a minimum of 5 ml saliva. All participants in the case and control group were asked not to eat, drink or brush their teeth, smoke at least one hour prior to the trial, and not consume any alcoholic beverages 24 hours prior to collection¹. The authors confirm that all methods were performed in accordance with the relevant guidelines and regulations.

Evaluation of the presence of HPV with MY09 / 11 primer

Genomic DNA was extracted from saliva samples of the case (HNSCC patients) and control group using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) as directed by the manufacturer. The DNA concentration was measured using the ND-1000 Nanodrop at 260/280 nm. Furthermore, the DNA integrity was tested using a 1.5 percent agarose gel and kept at 20 °C for future study. The presence of HPV in the saliva was determined using the MY09 / MY11 oligonucleotide primers 5'-CGT CCM ARR GGA WAC TGA TC-3' and 5'-CGT CAG GGW CAT AAY AAT GG-3', which amplify a region of about 450 pb in the genome of most HVP types¹⁶. A 25 L reaction mixture containing 10 pmol of each primer, 50 M of each dNTP, 200 ng of DNA template, 2 U of Taq DNA polymerase, 20 mM Tris-HCl, and 3 mM MgCl₂ was used for the PCR process. The PCR was started with a 5 minute denaturation at 95 °C, followed by 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 55 °C, and extension for 30 seconds at 72 °C, with a final extension at 72 °C for 7 minutes. A negative PCR control, consisting of a reaction mixture devoid of template DNA, was added to each pair of PCR reactions. Electrophoresis on a 1 percent agarose gel was used to examine the PCR results.

Variant analysis of HPV-16 E6 and E7 genes

PCR was used to examine the HPV-16 E6 and E7 genes, with an amplicon size of 762 bp targeted (Table 1). A 50 L reaction mixture containing 10 pmol of each primer, 50 M of each dNTP, 200 ng of DNA template, 2 U of Taq DNA polymerase, 20 mM Tris-HCl, and 3 mM MgCl₂ was used for the PCR process. An initial denaturation of 5 minutes at 95 °C was followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 50 seconds, extension at 72 °C for 50 seconds, and final elongation at 72 °C for 10 minutes. A negative control, consisting of a reaction mixture devoid of template DNA, was added to each pair of PCR reactions. Electrophoresis on a 1 percent agarose gel was used to examine the PCR results.

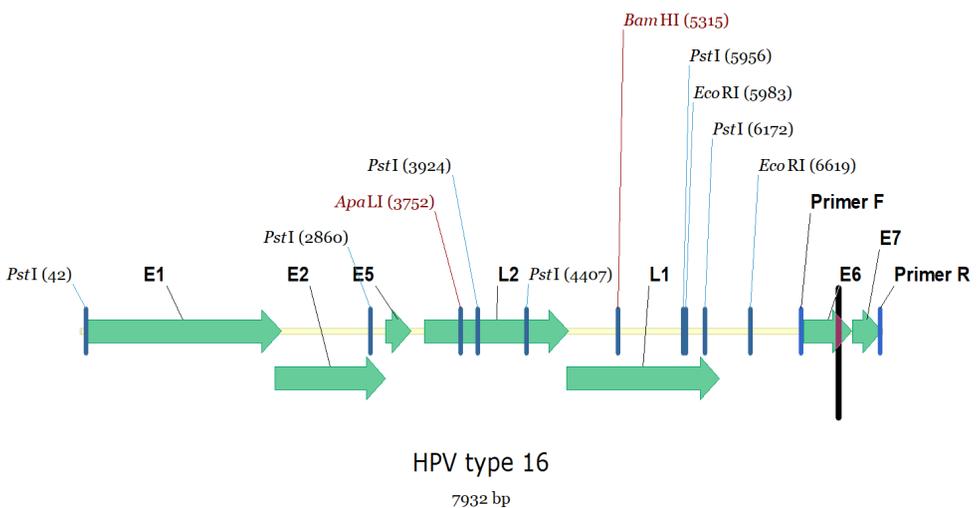
Variant analysis of HPV-16 L1 gene

HPV-16 L1 gene was analyzed by PCR (Table 1), targeting an amplicon size of 7932 bp.

Table 1. Primers for PCR analyzing of HPV-16 E6, E7 and L1 and HPV-18 E6, E7 and L1

Primer sequences	Genes
5'-CGA AAC CGG TTA GTA TAA AAG CAG AC-3'	HPV-16 E6
5'-TAG ATT ATG GTT TCT GAG AAC A-3'	HPV-16 E7
5'-ATG TGC CTG TAT ACA CGG GTC-3'	HPV-16 L1, Forward
5'-TTA CTT CCT GGC ACG TAC ACG C	HPV-16 L1, Reverse
5'-ATG GCG CGC TTT GAG GAT CC -3'	HPV-18 E6
5'- TTA CTG CTG GGA TGC ACA CC -3'	HPV-18 E7
5'- ATG TGC CTG TAT ACA CGG GTC -3'	HPV-18 L1, Forward
5'- TTA CTT CCT GGC ACG TAC ACG C -3'	HPV-18 L1, Reverse

PCR reaction method is similar to that described for HPV-16 E6 and E7 genes, and the schematic of HPV-16 E6 primers design is depicted in Figure 1.

**Figure 1.** Schematic primers design for PCR analyzing of HPV-16 E6.

Variant analysis of HPV-18 E6 and E7 genes

The HPV-18 E6 and E7 genes were amplified using polymerase chain reaction (PCR) with an amplicon size of 835 base pairs as the target (Table 2). A 50- μ L reaction mixture containing 10 pmol of each primer, 50 μ M of each dNTP, 200 ng of DNA template, 2 U of Taq DNA polymerase, 20 mM Tris-HCl, and 3 mM MgCl₂ was used for the PCR process. The primers were desaturated for 5 minutes at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 50 seconds, extension at 72 °C for 50 seconds, and final elongation at 72 °C for 10 minutes. Each set of PCR reactions contained a negative control consisting of a reaction mixture devoid of template DNA. Electrophoresis on a 1% agarose gel was used to verify the PCR results.

Variant analysis of HPV-18 L1 gene

The HPV-18 L1 gene was examined using a PCR assay with an amplicon size of 7857 bp (Table 1). The PCR reaction technique is identical to that described previously for the HPV-16 E6 and E7 genes, and a schematic primer design is shown in Figure 2 for reference.

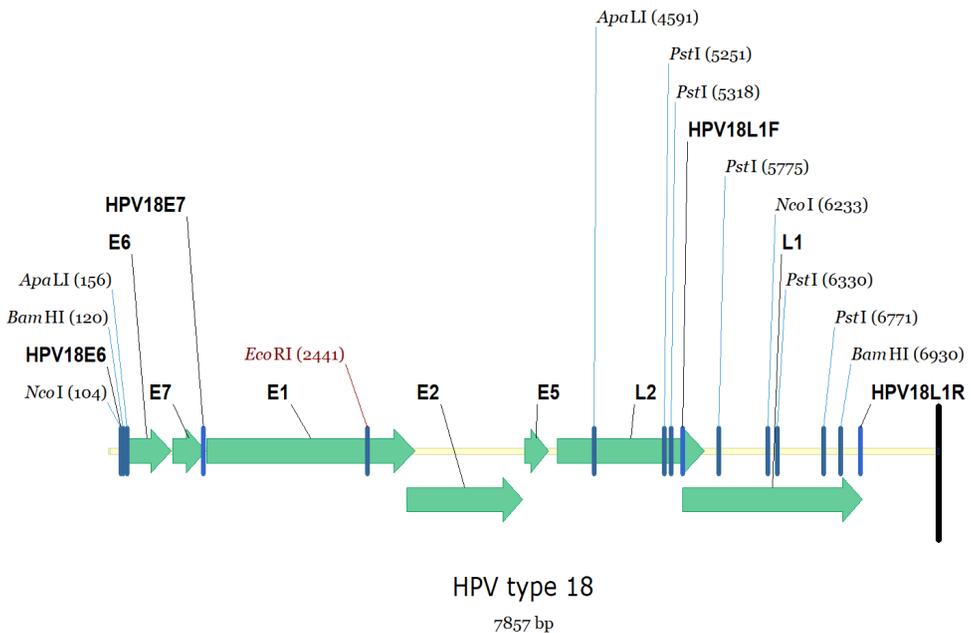


Figure 2. Schematic primers design for PCR analyzing of HPV-18 L1, R.

ABI Prism 377 sequencer Genetic Analyzer equipment (Applied Biosystems Foster City, Canada) was used to sequence all of the PCR products from the E6 and E7 genes to evaluate the HPV-16 and HPV-18 variants. The sequences were run according to the Big-Dye Terminator protocol (Applied Biosystems) (GeneAll, Seoul, South Korea). It was determined that the reference sequences were unique to each lineage and sublineage by aligning them to all previously known lineages and sublineages of A1-4, B1-4, C2-4, and D1-3¹⁶. The maximum likelihood approach was employed to build the phylogenetic tree, which was done using Mega software version 6. In order to analyze the HPV-18 lineages and sublineages, all HPV-18 sequences were analyzed using the Mega software version, which was used to depict the phylogenetic tree according to the maximum likelihood method with the reliability of bootstrap on 1000 replicates using the maximum likelihood method with bootstrap reliability on 1000 replicates. The A1-5, C, and B1-3 reference sequences for HPV-18 were also obtained from the GenBank database, as were the lineage and sublineage-specific reference sequences for HPV-18.

Results

This research comprised 30 people diagnosed with HPV-DNA over a year (from March 2020 to March 2021). Ten patients were male and five were female in the patient group. The control group consisted of 9 males and 6 females. The mean age and standard deviation (SD) of patient and control group were 53.84 ± 13.61 and 45.23 ± 12.80 , respectively. Four patients in the HNSCC group had tongue SCC, six had oropharyngeal SCC, three had soft palate SCC, and two had laryngeal SCC.

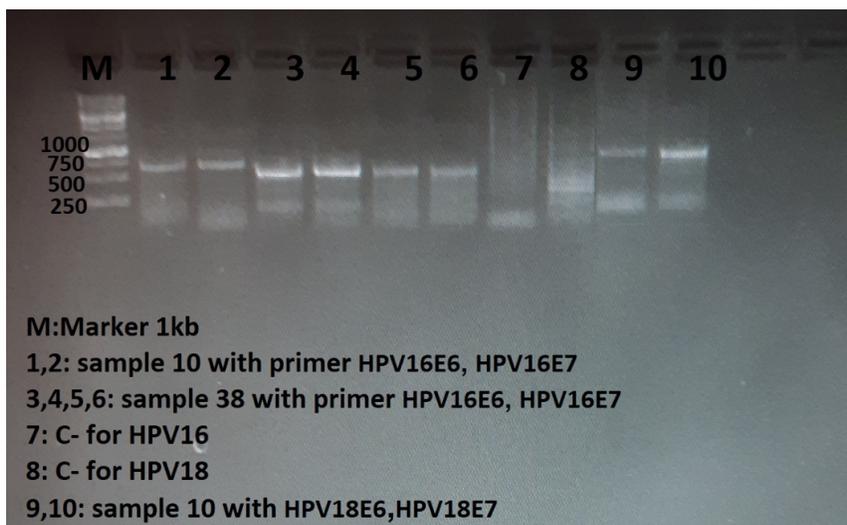


Figure 3. HPV-16 and HPV-18 (E6, E7) were sequenced in genomes isolated from saliva of subjects.

When the sequences recovered in this research were compared to the sequences of samples in the HPV-18 E6, E7, and L1 gene banks, it was discovered that the viral strain isolated from the patient's saliva belonged to lineage A3 (Figure 4-D, E, F). Overall, lineages A1 of HPV-16 (Figure A, B, C) and A3 of HPV-18 variations (Figure 4-D, E, F) were discovered (Figure 4), as shown by the red outlined data.

Discussion

HNSCC is a group of heterogenic cancers with multiple risk factors. HPV positivity may be an important contributing factor in the histologic grade and prognosis of cases with HNSCC². Risk factors of HPV transmission include multiple sex partners, tobacco consumption, and HIV infection. The roots of HPV transmission include sexual or nonsexual contact with the affected person, transmission through saliva contaminated with the viruses, breastfeeding, transmission during labor³. In the elderly, the most important predisposing factors for HNSCC are alcohol and tobacco use, but in younger people, the role of alcohol, and tobacco in HNSCC is lower and the number of young people with HPV are more than older ones. Hence, the HPV virus may be one of the major risk factors for HNSCC in the young population⁴. The annual incidence of oropharyngeal SCC was increased, while SCC incidence in the other sites of oral cavity was decreased. These differences may be attributed to the role of HPV in HNSCC pathogenesis⁵.

The role of HPV virus in anogenital cancers is confirmed. Furthermore, HPV has a role in 20 – 40 % of HNSCC⁶. PCR test to detect the expression of E6 and E7 oncogenes in HPV virus is the best diagnostic test for detection of HPV in HNSCC⁷. The carcinogenic's mechanism of HNSCC is attributed to E6 and E7 oncogenes. HPV-E7 protein disrupts the pRb-mediated cell-cycle control and causes a reduction in cyclin D1 synthesis and the over-expression of the cyclin-dependent kinase 4/6 inhibitor p16^{INK4A}. HPV E6 protein promotes the destruction of P53 tumor suppressor gene⁸. HPV positive HNSCC patients have a better prognosis than HPV negative patients, and their response to treatment is better⁹. Some studies suggested that HPV-18 is the dominant type of HPV in Iranian HNSCC patients, but these reports are controversial and this controversy attribute to the geographic, cultural, and habit difference^{10,11}. Wood et al.¹³ showed that the prevalence of oral HPV varied significantly in different geographical areas. Seifi et al.¹¹ introduced HPV-18 as the dominant type of virus in East Azerbaijan province of Iran. Delavarian et al.¹⁴ found no correlation between oral SCC and HPV infection. These results were in line with Sibers et al.¹² and Sisk et al.¹⁵ but in contrast with Kreimer et al.¹⁷. A systematic review study by Jalilvand et al. stated that HPV was detected in 44.4% of patients with head and neck cancers¹⁶. The most common HPV types were HPV-16 and -18. There was a strong association between HPV-16 and oropharyngeal cancer. These studies reflect the controversial reports about HPV prevalence and its subtypes in Iran. This controversy recently confirmed by a systematic review study¹⁸.

In conclusion, the HPV vaccine is available for the prevention of cervical cancer. Gardasil is a type of HPV vaccine which induces immunity against HPV-6, -11, -16, -18. Clinical trials on the effectiveness of this vaccine showed over 98% efficacy in the prevention of cervical, anal, vulvar, and vaginal cancers¹⁹. Genomic analysis of HPV in the saliva of HNSCC patients in the present study is a starting point for designing a vaccine to prevent HPV-associated HNSCC.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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Data availability

Datasets related to this article will be available upon request to the corresponding author.

Authors Contribution

MK and MD conceived the study idea and led data collection. MK, MAN, and MD created the study protocol and wrote the original draft. MAN and SM contributed in collecting the saliva sample. MD, MK and MAN contributed to data analysis / interpretation and preparation of the manuscript. MK, MAN and SM led the writing-review & editing. MK and MD interpreted the results. All authors read and approved the final manuscript.

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