Original Article

Detection of human papilloma virus-E6/E7 proteins of high-risk human papilloma virus in saliva and lesional tissue of oral squamous cell carcinoma patients using nested multiplex polymerase chain reaction: A comparative study

Rajbir Kaur Grewal[1], Keya Sircar[2], Kishore G Bhat[3], Dilpreet Singh Grewal[4], Krishan Kumar Tyagi[5], Simon David[6]

Departments of [1]Oral Pathology and Microbiology and [4]Prosthodontics, Desh Bhagat Dental College and Hospital, Mandi Gobindgarh, Fatehgarh Sahib, [2]Department of Oral Pathology and Microbiology, Faculty of Dentistry, Jamia Millia Islamia, New Delhi, [3]Department of Immunology and Microbiology, Maratha Mandal's Nathajirao G Halgekar Institute of Dental Sciences and Research, Belgaum, Karnataka, India, [5]Department of Oral Pathology and Microbiology, M. B. Kedia Dental College, Birgunj, Nepal, [6]General Dental Practitioner, Ferozepur, Punjab, India

Abstract

Introduction: Human papilloma virus (HPV)-associated oral squamous cell carcinoma (OSCC) shows different biological behavior as compared to tobacco-induced OSCC. Mere presence of HPV in OSCC is of no clinical significance; however, the integration of HPV-DNA through E6/E7 gene into the host genome is important as it affects the development and progression of OSCC.

Aim: The aim of this study was to determine the presence of E6/E7 proteins of high-risk (HR) HPV (HPV16 and HPV18) in saliva as well as lesional tissue of OSCC patients and to determine the use of saliva as an alternative to tissue for E6 and E7 proteins in OSCC.

Materials and Methods: Histopathologically confirmed 47 cases of OSCC were taken up for the study. The tumor tissue and saliva sample of each patient were obtained to detect the presence of HPV16 and HPV18 along with E6/E7 proteins in both samples by nested multiplex polymerase chain reaction (NMPCR). The data were analyzed using Student *t*-test (2 tailed) and Wilcoxon signed-ranks test.

Results: In tumor tissue, 40.42% of cases showed HPV16 (19/47) positivity while 34.04% were HPV18 (16/47) positive; whereas, in salivary sample, 31.91% showed HPV16 (15/47) positivity while 25.53% of cases were HPV18 positive (12/47). Mean age of participants was 46.7 years, males showed no significant difference from females in the prevalence of HPV 16/18 with tongue being the most common site for the occurrence. There was no statistically significant difference for HPV16/18 presence in tissue and saliva sample of OSCC. Taking lesional tissue sample as standard, sensitivity and specificity for HPV16 and HPV18 in saliva by NMPCR was estimated at 68.42% and 92.86%, respectively. The accuracy level of NMPCR detection for HPV16 was 82.98% and HPV18 was 65.96%.

Address for correspondence: Dr. Rajbir Kaur Grewal, 286 Model Gram, Ludhiana - 141 002, Punjab, India.
E-mail: drrajbirgrewal@gmail.com
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Conclusion: The study revealed no significant difference in the prevalence of HPV (16/18) among tissue and saliva of OSCC patients in Indian population. The study also found no difference in the level of DNA content of HPV in saliva and tissue indicating that saliva can be used as an alternative predictor of HPV positivity in OSCC.

Keywords: Oncogenic virus, orogenital, prognosis, saliva

INTRODUCTION

DNA viruses such as human papilloma virus (HPV) is well recognized to play a role in the initiation or development of oral squamous cell carcinoma (OSCC).[1,2] The International Agency of Research on Cancer[3] declared HPV16 and HPV18 (High-risk [HR] HPV) as human carcinogens and are also responsible for the most HPV-caused cancers. HPV-positive squamous cell carcinoma(SCC) show clinically distinct behavior from HPV-negative SCC as patients with HPV-positive SCC have a better prognosis than HPV negative.[4,5]

HPV DNA consists of nine open-reading frame sequences with seven early and two late-phase genes (E1–E7, L1–L2). HPV integration into the tissue occurs through viral episomal DNA rupture with preservation of E6 and E7 segments which undergo transcription causing disruption of cell cycle regulators. E6 degrades p53 oncoproteins and E7 binds to the Rb oncoprotein, causing abnormal cell growth, inhibition of apoptosis and dysregulation of the cell cycle. These early genes E6 and E7 can be easily detected in tissues and body fluids.[6,7] Therefore, the evaluation of E6 and E7 proteins of HR-HPV in saliva could prove to be useful diagnostic tool in OSCC.

Syrjänen *et al.* in 1983 first proposed participation of HPV in SCC.[8] However, recent evidence has linked that orogenital sexual contact can lead to transmission of HPV16 and HPV18 to the oral cavity which initiated many studies on HPV16 and 18 detection in OSCC.[9,10]

The reported prevalence of HPV varies broadly[11,12] which has been attributed to factors such as type of specimen collected, method employed for detection of HPV and geographic locations.[13] Indian studies evaluating HPV16 and 18 in paraffin-embedded lesional tissue samples of OSCC reported prevalence ranging from 20% to 50%.[14]

There is a growing group of young adults and women OSCC patients with no history of tobacco or alcohol consumption. Gradually, research has focused on identifying potential viral etiologic factors such as oncogenic HPV in such patients due to orogenital sexual contact with the transmission of HPV specially HPV16/18 from the genital area to oral cavity which may be attributed to new lifestyle habits.[12] In 2007, the World Health Organization stated that HPV was a causative factor for oral cancers.[15]

Syrjänen and Syrjänen demonstrated that HPV is present, more in the superficial epithelial cell layers than in the basal cell layer. These virus-infected superficial cells are shed into the saliva. Thus, saliva can be successfully used to detect the presence of HPV.[16] It has also been reported that the early detection of HPV in OSCC has been associated with more survival rate and better response to chemotherapy.[17] Several studies have been done to detect the presence of HPV in lesional tissue samples of OSCC as well as in salivary rinses from patients with OSCC.[18,19]

The HPV16 and 18 involvement in OSCC is supported by many authors[16,18,20] on the basis of subsequent evidence: (1) the well-assessed broad epitheliotropism of HPV,[21] (2) morphological similarities between oropharyngeal and genital epithelia,[22] (3) ability of immortalizing human oral keratinocytes *in vitro*,[15] and (4) the strongly established etiological role of HR-HPV in cervical cancer.[23]

However, a thorough review of available English literature revealed only anecdotal studies detecting HPV in lesional tissue and saliva sample of the same OSCC patients.[18,19,24] Therefore, the study was conducted with the aim to determine the prevalence of HR-HPV HPV16 and HPV18 in lesional and salivary sample of OSCC patients and also to determine the use of saliva as alternative to tissue for the presence of HPV16, 18 along with E6 and E7 proteins in OSCC.

MATERIALS AND METHODS

This cross-sectional study was conducted from October 2011 to January 2014 in collaboration with the Department of Immunology and Microbiology of Maratha Mandal's Nathajirao G Halgekar Institute of Dental Sciences and Research, Belgaum. The study was reviewed and approved by the Ethical Committee of Institute.

Sample selection criteria

Only those patients were selected for the study that had not undergone any surgical treatment, radiotherapy, chemotherapy and no history of tobacco habit. Patients with a history of any earlier treatment or patients with

recurrent OSCC were excluded from the study. Saliva sample with minimum 2–5 μg of DNA content was only further taken up for study.[18]

During the study, 182 patients with OSCC were examined for the study, 34 patients had a previous history of surgical treatment, radiotherapy or chemotherapy for OSCC, 87 patients had previous history of tobacco and alcohol consumption, thus were excluded from the study. A total of six patients refused to participate in the study, and eight salivary samples yielded DNA content <1 μg hence could not be taken up for the study. Consequently, only 47 histopathologically confirmed cases of OSCC were selected during the study, after the application of inclusion and Exclusion criteria in schematic manner [Figure 1].

After obtaining informed written consent from the patient, incisional biopsy was done for clinically suspected cases of OSCC. Subsequent to histopathological confirmation of biopsy for OSCC, saliva sample was collected from the same patient before commencement of any treatment.

Tissue sample collection

The biopsy tissue was stored in 10% neutral buffered formalin. On the receipt of tissue in the laboratory, tissue was processed and later formalin-fixed paraffin-embedded blocks were prepared, from which 5–6 paraffin sections (5 μm each) were later collected in wide mouth sterile plastic container that was later dewaxed. DNA extraction was done using modified proteinase K method. After DNA extraction, DNA was stored at −80°C till further analyzed.

Saliva sample collection

The saliva of patients was collected on the second visit after incisional biopsy confirmed of OSCC. Patients were asked to spit saliva into sterile wide-mouth plastic container.[18] Later, with the help of sterile plastic dropper, 1 ml of saliva was taken and mixed with 1.5 ml of tris-EDTA buffer in Eppendorf tubes which was later sent to the laboratory. DNA extraction was done immediately using modified proteinase K method for saliva sample on the receipt of the samples in the laboratory and the extracted DNA was stored at −80°C till further analyzed. After DNA extraction was done (for both the saliva samples and the deparaffinized tissue samples), two cycles of nested multiplex polymerase chain reaction (NMPCR) were used to evaluate E6/E7 along with HPV 16/18.

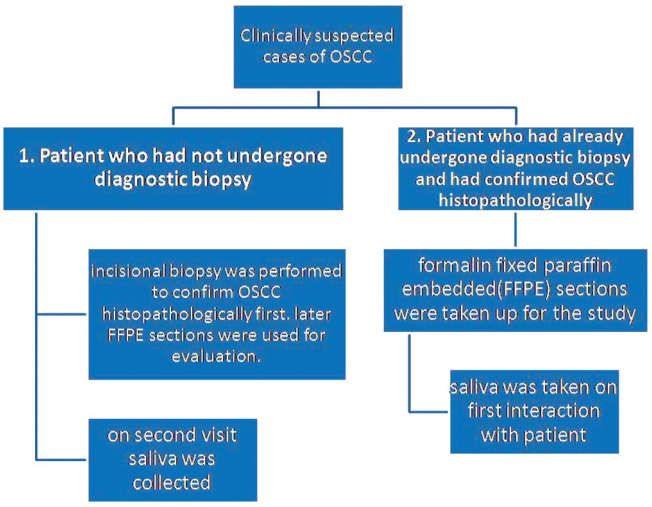

Figure 1: Selection of oral squamous cell carcinoma cases for the evaluation of human papilloma virus

To avoid bias, all the tissues were formalin fixed and embedded in paraffin as some of patients who had already undergone diagnostic biopsy only had formalin-fixed paraffin-embedded sections available, and the second biopsy in such cases would have caused more trauma to the patient. Therefore, only formalin-fixed paraffin-fixed tissues were used for evaluation.

Positive controls

Cervical HPV16 and HPV18 positive tissue was taken up as positive control. This was further validated by use of specific molecular weight band marker in all NMPCR cycles and comparing it with DNA ladder (total laboratory software, UK).

Negative control

Distilled water was used as negative control in all NMPCR cycles.

Polymerase chain reaction procedure

NMPCR procedure consisted of two PCR cycles. First, PCR cycle was done for evaluation of E6 and E7 proteins with the help of three primers (Bioserve Tech. Hyderabad, India), and second, PCR reaction was done for evaluation of HPV16 and 18 with the help of four primers [Table 1].

Polymerase chain reaction cycle I

Denaturation (94° C-3 min), 30 amplification cycles (94°C-1 min, 40° C-1 min, 72° C-1 min), followed by Elongation (72° C-5 min, 4° C-1 min). The length of amplicons generated by amplification with GP

Table 1: Sequence of primer used

Primer	Sequence (5'-3')	Length
GP E6 3F	GGGWGKKACTGAAATCGGT	19 Tm49
GP E7 5B	CTGAGCTGCARNTAATTGCTCA	23 Tm52
GP E7 6B	TCCTCTGAGTYGYCTAATTGCTC	23 Tm53
HPV 16-1	CACAGTTATGCACAGAGCTGC	21 Tm 54
HPV 16-2	CATATATTCATGCAATGTAGGTGTA	25 Tm51
HPV 18-1	CACTTCACTGCAAGACATAGA	21 Tm50
HPV 18-2	GTTGTGAAATCGTCGTTTTTCA	22 Tm49

E6/E7 Consensus primers ranged from 636 (HPV 16) to 674 (HPV18) bp.

Polymerase chain reaction cycle II

Amplified DNA (3 μl) which was obtained after PCR Cycle I was dispensed into respective PCR tubes for the PCR Cycle II. Denaturation (94° C-3 min), 30 amplification cycles (94° C-1 min, 56° C-1 min, 72° C-1 min), followed by Elongation (72° C-5 min, 4° C-1 min).

Gel electrophoresis

The amplified products were subjected to gel electrophoresis for band formation. The presence of positive band at 457 bp indicated the presence of HPV16 and a positive band at 322 bp indicated HPV18.[8]

The data thus collected were subjected to statistical analysis.

Statistical analysis

The SPSS software package version 17 was used for statistical analysis. Wilcoxon Signed-Ranks Test and Student's *t*-test was used to evaluate the difference, in total HPV prevalence and type-specific HPV prevalence between saliva and lesional tissue [Tables 2-6]. Student's *t*-test was used to evaluate the difference in number of DNA copies of HPV16 and 18 in saliva and lesional tissue of OSCC [Table 5]. Sensitivity and specificity of NMPCR for detection of HPV in saliva were calculated by taking lesional tissue as standard. The positive and negative predictive values were also calculated to evaluate the efficacy of sensitivity and specificity [Table 6].

RESULTS

In the study, the prevalence of HPV16, HPV18 in saliva and tissue [Table 2] showed no significant difference indicating that both saliva and tissue can be used for detection of HPV along with E6 and E7 proteins.

In the present study, age range varied from 28 to 72 years with mean age of 46.74 years and the prevalence of HPV 16 and/or 18 showed no significant difference among males and females, respectively. On comparing histological differentiation of OSCC between saliva and tissue, no statistically significant difference was found among well, moderately and poorly differentiated OSCC, respectively [Table 4]. In the study, the tongue was the most common site. On the evaluation of DNA content [Table 5], the study showed that both saliva and tissue produced the same number of DNA copies and showed no significant difference, indicating that saliva can be used as an alternative diagnostic tool for evaluation of HPV. The positive predictive value of 86.25% and negative predictive value of 81.25% [Table 6], indicates that NMPCR and saliva can be used for detection of E6/E7 proteins along with HPV16/18 in OSCC.

Table 2: Total human papilloma virus 16/18 positivity

HPV type	Saliva (*n*=47), *n* (%)	Tissue (*n*=47), *n* (%)	*Z*	*P*
HPV 16 positive	15 (31.91)	19 (40.42)	−1.414	0.157*
HPV 18 positive	12 (25.53)	16 (34.04)	−1.000	0.317*
HPV (16+18) positive	4 (8.51)	12 (25.53)	3.000	0.003**

*Nonsignificant, **Significant, using Wilcoxon signed-ranks test.
HPV: Human papilloma virus

Table 3: Detailed information of patients along with human papilloma virus positivity

	HPV 16 positive		HPV 18 positive		HPV 16 and 18 positive	
	Saliva	Tissue	Saliva	Tissue	Saliva	Tissue
Total positive cases (*n*=47)	15	19	12	16	04	12
Gender						
Male (*n*=36)	12	15	11	14	3	10
Female (*n*=11)	3	4	1	2	1	2
Age (years)						
<40 (*n*=9)	4	5	2	4	2	4
40-60 (*n*=35)	10	13	8	10	2	8
>60 (*n*=3)	1	1	2	2	0	0
Differentiation						
Well (*n*=21)	6	9	7	9	3	7
Moderate (*n*=16)	6	7	4	6	1	5
Poor (*n*=10)	3	3	1	1	0	0
Site/location						
Buccal mucosa (*n*=9)	3	4	1	3	0	3
Tongue (*n*=23)	11	14	10	13	4	9
Alveolus (*n*=11)	1	1	1	0	0	0
Lip (*n*=4)	0	0	0	0	0	0

HPV: Human papilloma virus

Table 4: Correlation coefficient along level of significance

	HPV 16		HPV18		HPV16and 18	
	Saliva versus tissue		Saliva versus tissue		Saliva versus tissue	
	Z	*P*	*Z*	*P*	*Z*	*P*
Total positive cases	−1.414	0.157*	−1.000	0.317*	3.000	0.003**
Gender						
Male (*n*=36)	−1.134	0.257*	−1.069	0.285*	−2.828	0.005**
Female (*n*=11)	−1.000	0.317*	0.000	1.000*	−1.000	0.317*
Age (year)						
<40 (*n*=9)	−1.000	0.317*	−1.000	0.317*	−1.000	0.317*
40-60 (*n*=35)	−.816	0.414*	−0.302	0.763*	−2.646	0.008**
>60 (*n*=3)	−1.000	0.317*	−1.000	0.317*	−1.000	0.317*
Differentiation						
Well (*n*=21)	−1.000	0.317*	−0.816	0.414*	−1.732	0.083*
Moderate (*n*=16)	0.000	1.000*	0.000	1.000*	−2.000	0.46*
Poor (*n*=10)	−1.732	0.083*	−1.414	0.157*	1.414	0.157*
Site/location						
Buccal mucosa (*n*=9)	−1.00	0.317*	0.000	1.000*	0.000	1.000*
Tongue (*n*=23)	−1.134	0.257*	−2.111	0.035*	−3.000	0.003**
Alveolus (*n*=11)	0.000	1.000*	−1.000	0.317*	0.000	1.000*
Lip (*n*=4)	0.000	1.000*	−1.000	0.317*	0.000	1.000*

*Nonsignificant, **Significant at 1% of level, after application Wilcoxon signed-ranks test. HPV: Human papilloma virus

Table 5: Comparison of salivary and lesional tissue human papilloma virus DNA 16 and 18 content

Comparison	Mean (number of DNA copies)	SD	SEM	Lower limit	Upper limit	*t*	df	Significant (two-tailed)	Significance
Tissue HPV16 versus saliva HPV16	−207.692	12,114.004	3359.820	−8528.112	6112.727	−0.359	12	0.726	NS
Tissue HPV18 versus saliva HPV18	−966.667	11,897.339	4857.068	−13452.158	11,518.825	−0.199	5	0.850	NS

95% CI of the difference. SD: Standard deviation, SEM: Standard error of mean, df: Degree of freedom, NS: Nonsignificant, with application of Student t-test. HPV: Human papilloma virus, CI: Confidence interval

DISCUSSION

In the present study, a high specificity was observed as NMPCR technique was used, which amplifies the given target DNA twice, thereby eliminating the possibility of false-positive results. The sensitivity was moderate as even small amounts of DNA that were present in saliva sample were amplified in the first PCR reaction and later this amplified product was used for second PCR reaction, thereby giving more amount of DNA for evaluation.

Diverse studies have compared the detection methods such as ISH, PCR, Southern blot and found that PCR was more sensitive for detection for HPV in OSCC.[25,26] This can be attributed to two principal advantages of PCR. First, only small amounts of tissue are required for evaluation, and second, the specificity of DNA amplification is such that unrelated material in saliva is unlikely to affect the result.[27] Therefore, in the study, NMPCR technique was chosen as this facilitated in two ways first, different HPV type detection based on PCR product size, and second, direct detection of the viral oncogenes along with the extension of assay with multiplex primers cocktails for further HPV genotyping.[8]

In the study, the prevalence of HPV18 in lesional OSCC was found to be 25.53% which is similar to studies carried out by Wen *et al.* (29.4%) and Luo *et al.* (30.4%). Therefore, the present study showed lower prevalence of HPV18 as compared to HPV16 in HNSCC.[28,29]

The prevalence of HPV in saliva in the present study was 40.42% (19/47), HPV 16 (31.91%), and HPV 18 (25.53%). Similar rates of HPV prevalence in the saliva of patients of OSCC were reported by SahebJamee *et al.* (40.9%) and Chuang *et al.* analyzed HPV16, E6 and E7 DNA copy number in salivary rinses of OSCC and found 33.9% prevalence of HPV 16.[18,30] Similarly, Adamopoulou *et al.* also analyzed saliva samples OSCC for HR-HPV and reported the prevalence of 10.3% out of which 50% was HPV 16.[31] Zhao *et al.*[19] used PCR to detect HPV16, E6 and E7 DNA level in primary tumors and salivary rinses from patients with HNSCC and reported total of 45.6% of primary HNSCC and 32.6% of saliva rinse samples from HNSCC patients had detectable HPV16 DNA.

Chuang *et al.*[18] who assessed HPV-DNA shed postoperatively into the saliva in HNSCC concluded that HPV16 presence in follow-up salivary rinses preceded clinical detection of disease recurrence by an average of 3.5 months and patients with the presence of HPV16 DNA in surveillance salivary rinses are at significant risk for recurrence. Thus, they concluded that the quantitative measurement of salivary HPV16 DNA has promise for surveillance and early detection of recurrence.

In the present study, with the level of prevalence of HPV in saliva of participants, both the predictive values indicate that test results are correct in detecting HPV whether positive or negative. Therefore, indicating that use of saliva for detection of HPV with the help of NMPCR is likely to give more accurate results whether positive or negative. Thereby, indicating that saliva can be used as reliable tool for assessing HPV status of the patient in OSCC.

In the study, some cases were positive for HPV in both tissue and saliva sample (31.9%) on the contrary some cases were only positive in saliva or tissue sample alone. The positivity of saliva alone or lesional tissue alone could be explained due to the two different cellular localizations of HPV DNA replication, i.e., in the germinative and superficial epithelial layers. During the germinative stage, replication is localized in the cells of the lower portion of the epithelium including the basal cells, ensuring a persistent, and latent infection of the basal epithelial cells leading to detection in lesional tissue.[14,32-34] The second phase of vegetative replication leads to shedding of HPV into superficial cells and thereby, its consequent shedding into saliva.[18] This fact is further supported by Syrjänen *et al.*[8] who demonstrated that the presence of HPV in epithelium increased progressively from the basal cell layer (5.8%) to the superficial layers (100%).Thus, the presence of HPV in OSCC may be elicited by its detection in lesional tissue and/or in saliva sample of the patient.

However, the study has few limitations such as patient follow-up could not be assessed to determine the prognosis of the HPV-positive OSCC as compared to HPV-negative OSCC and comparison of different isolation techniques for HPV16/18 could not be done. Subsequently, future prospects of the study emphasize on seroepidemiologic

Table 6: Sensitivity and specificity of polymerase chain reaction of saliva for human papilloma virus 16/18

Saliva (*n*=47)	True positive	False positive	True negative	False negative	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
HPV 16	13	2	26	6	68.42	92.86	86.67	81.25	82.98
HPV 18	6	6	25	10	37.50	80.65	50.00	71.43	65.96
HPV 16+18	4	0	34	9	30.77	100.00	100.00	79.07	80.85

HPV: Human papilloma virus

studies that required to study the relationship between incident squamous epithelial lesions such as leukoplakia, lichen planus or OSCC and HR-HPV. For further confirmation of the prevalence of HPV in head-and-neck malignancies, large population studies are necessary in an assortment of clinical settings.

CONCLUSION

The study revealed the prevalence of HPV (16/18) along with E6/E7 proteins in OSCC in the Indian population, which is suggestive of its role in oral carcinogenesis. Since no significant difference in DNA content of HPV16/18 was found in lesional tissue and saliva, the presence of HPV16/18 in saliva can be used reliably as predictor for HPV positivity in OSCC and also good usability in a routine HPV diagnosis, thereby providing better prognosis and specifically targeted chemotherapy for HPV proteins in such patients. The presence of high-risk HPV16/18 genotypes in saliva of OSCC patients may be used to identify patients who could be at HR to develop recurrent OSCC to submit to strict follow-up, primary and secondary prevention.

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Conflicts of interest
There are no conflicts of interest.

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