ORIGINAL ARTICLE

HPV involvement in OSCC: Correlation of PCR results with light microscopic features

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ABSTRACT

Objectives: The study evaluated pathognomic histopathological features with the help of light microscopy for detecting the integration of human papillomavirus (HPV) (type 16 and 18) in oral squamous cell carcinoma (OSCC). **Materials and Methods:** Forty-five histopathologically diagnosed cases of OSCC were evaluated for the presence of E6/E7 protein of HPV (16 + 18) with the help of nested multiplex polymerase chain reaction. Both HPV-positive and -negative cases were evaluated for four histological features: Koilocytes, dyskeratosis, invasion, and alteration of collagen. **Results:** Fischer's exact test showed significant difference ($P < 0.01\%$) for the presence of koilocytes and dyskeratosis, whereas no difference was observed for invasion and alteration in collagen between HPV-positive and -negative OSCC. **Conclusion:** The presence of koilocytes and dyskeratosis at light microscopic level can be used as a marker for the presence of HPV (type 16 and 18) in OSCC.

Key words: Dyskeratosis, human papillomavirus , histopathology, koilocytes, oral squamous cell carcinoma, polymerase chain reaction

INTRODUCTION

Oral squamous cell carcinomas (OSCCs) are characterized by multiphasic and multifactorial etiopathogenesis. Tobacco and alcohol are the most common risk factors for oral malignancy. Other factors, including DNA viruses, especially human papilloma virus (HPV) have been documented to play a role in the initiation or development of these lesions.[1]

HPV involvement in OSCC was first proposed in 1983 by Syrjanen *et al*. and then supported by others on basis of the fact that HPV shows epitheliotropism and has the ability to immortalize human oral keratinocytes *in vitro*.[2] High-risk HPV type 16 and 18 were declared as human carcinogens by the International Agency of Research on Cancer.[3]

The HPV family comprises more than 100 genotypes, classified in accordance with the type of epithelial cells infected and the ability to affect cellular transformation. Certain types of HPV such as HPV1 infect cutaneous epithelial cells, whereas HPV6, 11, 16, and 18 infect mucosal epithelial cells of the oral cavity, oropharynx, anogenital tract, and uterine cervix.[4] The genomic HPV DNA has nine open-reading frame sequences present on single strand of DNA. These are divided into seven early (E1–E7) and two late-phase genes (L1–L2). Expression of viral oncoproteins E6 and E7 interferes with crucial cellular mechanisms such as cell cycle regulation and apoptosis.[5]

HPV-positive SCC are clinically and molecularly distinct from HPV-negative SCC and may be associated with different prognostic outcomes.[6]

The prevalence of HPV has been assessed by many techniques, including polymerase chain reaction (PCR), southern blot, dot blotting, and *in situ* hybridization (ISH). These methods are satisfactory and reliable for detection of HPV, but are neither easily available universally, nor can every patient afford these procedures especially in developing countries such as India.

While histopathological analysis by light microscopy is the most commonly used method for confirming a diagnosis, it is also a useful method for observation of viral particles when molecular biology methods are not available. The purpose of the study was to compare the histopathological features

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with PCR method to predict the presence of HPV infection in OSCC biopsies as the detection of HPV in patients of OSCC may be of great help in planning targeted therapy and for better prognostication.

MATERIALS AND METHODS

The study was conducted at the Department of Oral Pathology and Microbiology. The study was reviewed and approved by an ethical board of the college.

The study sample comprised of 45 histologically confirmed cases of OSCC. Each case of OSCC was evaluated for the presence of E6 and E7 protein of HPV 16 and HPV 18 with the help of nested multiplex PCR.

Two sets of primers (Bioserve Tech. Hyderabad, India) were used in two successive PCR reactions. In the first reaction, one pair of primers was used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The second round of primers (internal) were located within the desired amplification product produced by the first round primers (external) so they specifically amplified required DNA fragment. The amplified products were then gel electrophoresed for band formation. The identification of HPV 16 was done by the presence of a positive band at 457 bp and HPV 18 was identified as positive band at 322 bp [Figure 1].

For histological evaluation, 5-µm thick sections were obtained from the same paraffin-embedded squamous cell carcinoma blocks and later stained with hematoxylin and eosin stain. All the 45 cases were evaluated for light microscopy histological features, such as presence of koilocytes, dyskeratosis (individual cell keratinization), invasion and alteration in collagen.

In this study, the presence of koilocytes in tissue was considered significant if more than five koilocytes per 10 high power fields (HPF) were seen in both neoplastic and non neoplastic areas (just surrounding the neoplastic area). Only if koilocytes were present in both the areas, it was considered positive [Figure 2]; positivity for dyskeratosis was considered to be the presence of more than three dyskeratotic cells per 10 HPF [Figure 3]. Invasion was considered to be positive if epithelial cells were found in the connective tissue with clear breach of basement membrane [Figure 4], and collagen alteration was considered when any abnormal pattern of collagen structure in connective tissue was found [Figure 5].

A single pathologist examined all the histological sections for the four features. The results of PCR evaluation for the presence of E6 and E7 protein of HPV 16 and HPV 18 were blinded from the pathologist assessing the histopathological slides in order to remove bias.

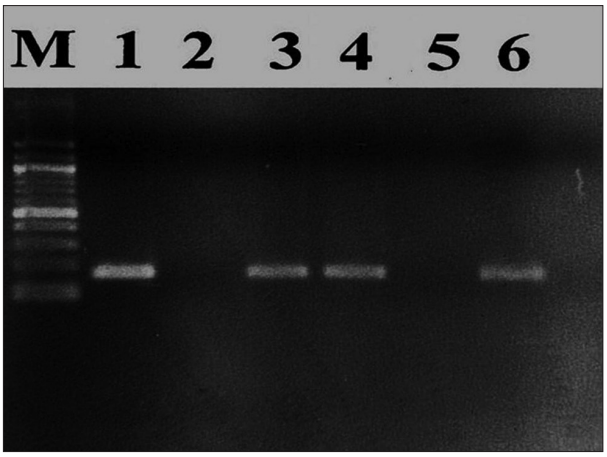

Figure 1: Band analysis: M lane: 100-bp deoxyribonucleic acid marker, lane 1: HPV 16-positive control (457 bp), lane 2: Human papilloma virus-negative control, lane 3: HPV 16-positive specimen, lane 4: HPV 18-positive control (322 bp), lane 5: HPV 16-negative specimen, lane 6: HPV 18-positive specimen

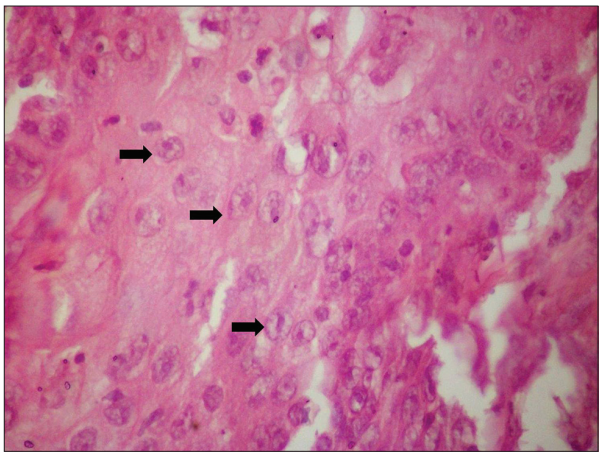

Figure 2: Koilocytes (black arrow) in epithelium (H&E, x400)

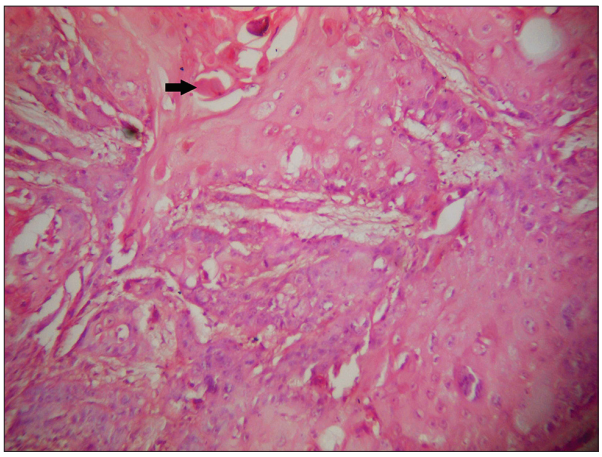

Figure 3: Dyskeratotic cells (black arrow) (H&E, x400)

Nested multiplex PCR revealed that out of 45 selected cases of OSCC, 22 cases (48.8%) were HPV16 positive, 13 cases (28.8%) were HPV18 positive, and 10 cases (22.2%) were positive for both HPV16 and 18.

Table 1 shows detailed clinicopathological data of patients' age, gender, site, and tumor differentiation of OSCC taken up for the study.

Two groups were made depending on the presence of HPV16, HPV 18, and both HPV16 and 18 separately for the four histological features [Table 2]. The data thus collected was subjected to statistical analysis. SPSS software package version 17 was used for statistical analysis.

RESULTS

Two-tailed Fischer's exact test was used for evaluating the difference for the four histological features between HPV positive and negative cases of OSCC.

Table 3 shows statistical evaluation of all four histological features in HPV16, HPV18, and combined, respectively.

When Fischer's exact test (two tailed) was applied for evaluating the difference in the presence of koilocytes it showed that there was a significant difference in the number of koilocytes in HPV positive and negative cases for HPV16, HPV 18 and combined HPV 16 and 18.

Dyskeratosis was significantly different in HPV 16 and combined HPV 16 and 18, but was not significantly different for HPV18. The other two histological features of invasion and abnormal alteration in collagen showed no significant difference between HPV positive and negative cases HPV16, HPV18, and combined HPV 16 18.

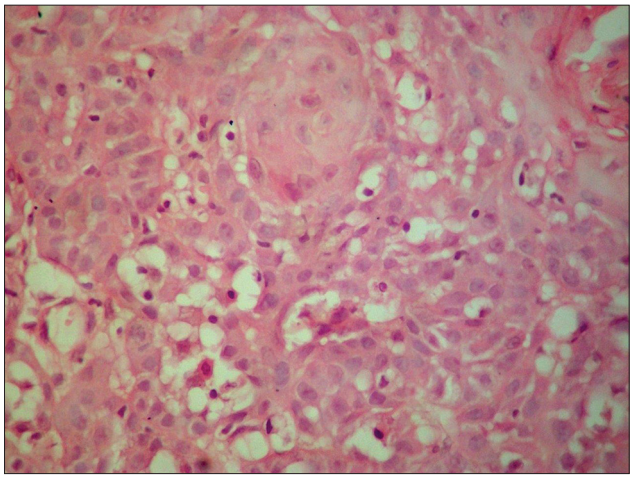

Figure 4: Invasion of epithelial cells in the connective tissue (H&E, x100)

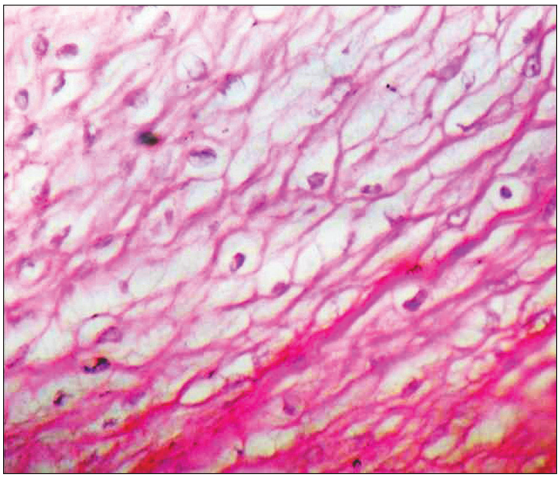

Figure 5: Abnormal pattern of collagen structure (H&E, x400)

Table 1: Clinicopathological data of patients

Positive HPV type (*n*=number of positive cases by PCR)	Gender		Age			Site				Grade		
	Male	Female	Less than 40 yrs	40-60	More than 60 yrs	Buccal mucosa	Tongue	Alveolus	Lip	Well differentiated	Moderately differentiated	Poorly differentiated
HPV 16 (*n*=22)	14	8	3	12	7	4	9	8	1	10	9	3
HPV 18 (*n*=13)	10	3	2	7	4	3	5	5	0	9	4	0
HPV 16+18 (*n*=10)	8	2	1	5	4	2	3	5	0	7	3	0

HPV: Human papillomavirus, PCR: Polymerase chain reaction

Table 2: Histological features according to HPV type

Feature	HPV 16 positive (*n*=22) (%)	HPV 16 negative (*n*=23) (%)	HPV 18 positive (*n*=13) (%)	HPV 18 negative (*n*=32) (%)	HPV 16 and 18 positive (*n*=10) (%)	HPV 16 and 18 negative (*n*=35) (%)
Presence of koilocytes	17 (77.27)	6 (26.08)	9 (69.23)	8 (25)	8 (80)	5 (14.28)
Presence of dyskeratotic cells	19 (86.36)	13 (56.52)	12 (92.30)	20 (62.5)	9 (90)	10 (28.57)
Invasion of epithelial cells into connective tissue	16 (72.72)	15 (65.21)	8 (61.53)	22 (68.75)	7 (70)	14 (40)
Abnormal pattern of collagen structure	15 (68.18)	15 (65.21)	7 (53.84)	21 (65.62)	6 (60)	14 (40)

HPV: Human papillomavirus

Table 4 shows the sensitivity and specificity of two histological features: Koilocytes and dyskeratosis when nested multiplex PCR is taken as gold standard procedure for identification of HR-HPV.

DISCUSSION

There have been numerous reports on HPV-DNA detection in HN-SCC with rates varying from 0% to 100% of carcinomas studied.[7,8] These differences in detection rate are due to at least two principal factors: (1) differences in the epidemiological distribution of oncogenic HR-HPVs in the world; and (2) different analytical methods used.[9,10]

The great variation in HPV prevalence found in OSCCs in different studies may be not only due to the differences among the analyzed population, but also due to the differences in the samples tested (ie, formalin-fixed or fresh biopsies, exfoliated fresh cells), the methods of DNA extraction, and most importantly the HPV detection methods used.[11,12] Although HR-HPV detection is important in clinical settings for the OSCC patients, there is no consensus on which to consider the "golden standard" among the numerous detection methods available either as single test or combinations.[13-16]

A few Indian studies have been done on the prevalence of HPV in OSCC in Indian population. The overall prevalence of HPV in OSCC in India has been reported as ranging from 20% to 50%.[17] The prevalence of HPV in OSCC also shows regional variation. It has been reported as 33.6% in Eastern India, 67% in South India, and 15% in Western India.[18] Chocolatewala *et al*., reported a prevalence of 17.6%-41.8% for HPV16, and 0%-47.3% for HPV18 in OSCC cases.[17]

Table 3: Application of Fischer's exact test (two tailed)

Histological feature	*P* value for HPV 16	*P* value for HPV 18	*P* value for HPV 16 and 18
Presence of koilocyte	0.0083*	0.0083*	0.0002*
Presence of dyskeratotic cells	0.0472*	0.0702	0.0008*
Invasion of epithelial cells into connective tissue	0.7494	0.7325	0.1513
Abnormal pattern of collagen structure	1.0000	0.5114	0.3011

*Significant at 1% level of significance, HPV: Human papillomavirus

Table 4: Sensitivity and specificity of koilocyte and dyskeratosis

HPV Type	Koilocyte		Dyskeratosis	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
HPV 16	77.27	73.91	86.36	43.47
HPV 18	52.94	14.28	92.30	37.5
HPV 16+18	80	85.71	90	71.42

HPV: Human papillomavirus

Nested amplification of the GP-E6/E7 PCR products with type-specific primers, was chosen to achieve exact typing of the HPV infections. These primers were again selected from sequence alignments of the E6/E7 genes, which were aimed to indicate sequence variation between closely related genotypes. In order to reduce the number of nested PCR for detection of two different genotypes of HPV, multiplex primer cocktails were used in which requirements for both the type-specific primers are met. This strategy allows first, HPV genotyping based on PCR product size; second, extension of assay with multiplex primers cocktails for additional HPV genotypes and also direct detection of the viral oncogenes.[19,20]

Total prevalence of HPV in lesional tissue of oral cavity proper was found to be 55.55%, HPV16 was found to be 48.88%, and HPV18 was found to be 28.88%. The high prevalence of HPV in present study may be due to the fact that most cases were from region of tongue, which is known to show high prevalence of High risk-Human papilloma virus in the oral cavity.

In the present study, the prevalence of HPV16 in SCC of oral cavity proper was found to be 48.88%, which is in accordance with a Mexican patient cohort study done by Anaya-Saavedra *et al*.,[14] which showed a high frequency of HPV positivity with prevalence of 43.5% in OSCC and Higa *et al*.,[21] who reported prevalence of 52.2%. Ostwald *et al*.[22] conducted a study for presence of HPV-DNA in head and neck squamous cell carcinoma and normal mucosa using PCR where they found 45% prevalence of HPV 16. Sugiyama *et al*.[23] studied the prevalence of HPV by PCR in OSCC, found 35% prevalence of HPV 16. In India, Balaram *et al*.[18] conducted a study on HPV in OSCC using PCR and found 41.8% prevalence of HPV 16.

The histological diagnosis of HPV presence in a given tissue sample, is based on HPV-related histopathological aspects, such as koilocytes, dyskeratosis, papillomatosis, hyperkeratosis, acanthosis, and parakeratosis. Koilocytes – consisting of the presence of abnormal cells that are vacuolated, with nucleus showing pyknosis and large clear perinuclear halos that usually occupies a greater volume than that of the cytoplasm –is the most common HPV-related cytopathic effect and is considered by pathologists to be the major histopathological feature for determination of HPV infection. It is considered as a pathognomonic sign of HPV-associated lesions.[24] Although some authors suggested an association between cytological and immunohistochemical positivity for HPV, others found no morphological aspects that confirmed the presence of HPV by PCR.[25]

Miyahara *et al*. (2011)[26] compared the histopathological analysis with PCR to predict the presence of HPV infection in OSCC biopsy tissues and found that the presence of koilocytes is unreliable for the detection of HPV presence in oral and oropharyngeal SCC.

Our study revealed that there was statistically significant differences for koilocytes and dyskeratosis between HPV (16,18) positive and negative cases, which indicated that koilocytosis and dyskeratosis can be reliably used as markers for the diagnosis of HPV in SCC.

This study also revealed that invasion and abnormal alteration in collagen structure showed no histological difference between HPV (16,18) positive and negative cases. Branca *et al*.[27] studied the difference in invasion pattern between HR-HPV positive and negative cases with the help of important regulators of cancer invasion and metastasis, such as matrix metalloproteinase-2 (MMP-2) and its tissue inhibitor (TIMP-2), which revealed that there was no significant difference between them. Similarly, Garzetti *et al*.[28] described marked overexpression of MMP-2 in microinvasive carcinomas as compared with cervical intraepithelial neoplasia, but this upregulation was unrelated to the HPV status in these lesions. Hence, even in our study we did not find any difference in invasion pattern between HPV-positive and -negative cases with the help of light microscope.

In the present study, when NMPCR is taken as gold standard, koilocyte shows high sensitivity of 77.27% with 73.91% specificity in detection of OSCC, whereas dyskeratosis shows sensitivity of 86.36% and specificity of 43.47% for HPV 16 alone.

In contrast to present study, Pannone *et al*.[16] used p16-IHC to demonstrate the presence of HPV and demonstrated that although p16-IHC is a good prognostic indicator when used in combination with HPV-DNA molecular methods, it is not satisfactory when evaluated as HPV detecting test when used alone (specificity less than 75%). They also showed that adding ISH technique, (although it is a method known to be less sensitive than PCR-based ones) has the advantage to preserve the morphological context of HPV-DNA signals in formalin fixed paraffin embedded samples and, unexpectedly, increased the sensitivity of p16/consensus PCR combination.

Thus, it is evident that no single technique can be used as an indicator of HPV testing but it is combination of techniques, which helps in detecting and evaluating prognosis of HPV-related OSCC.

On large community screening examination for detection of OSCC, especially in the developing countries, where molecular detection methods are not easily available, light microscopic features might provide help in selecting patients for further molecular detection methods of HPV as HPV-related OSCC has better prognosis than smoking-related OSCC. Thus the study points the use of light microscopic features, such as koilocyte and dyskeratosis for positive prediction of HPV 16 and 18's presence in OSCC.

CONCLUSION

The study concludes that the presence of koilocytes and dyskeratosis at light microscopic level can be used as an indirect marker for the presence of HPV (16,18) in OSCC. However, features such as invasion and abnormal alteration in collagen cannot be used as predictors for HPV status. Therefore, for economically poor patients of OSCC, indirect determination of the HPV status using koilocytosis and dyskeratosis as indicators, may eliminate the need for more expensive HPV tests using PCR, as HPV-positive patients are reported to have better prognosis than HPV-negative cases.

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