Original Article

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Pathology Section

Analysis of Human Papilloma Virus 16/18 DNA and its Correlation with p16 Expression in Oral Cavity Squamous Cell Carcinoma in North-Eastern India: A Chromogenic in-situ Hybridization Based Study

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ABSTRACT

Background: The incidence of head and neck squamous cell carcinoma (HNSCC) is quite high in North Eastern India. Apart from the traditional risk factors like tobacco and alcohol consumption, human papilloma virus (HPV) is now considered an established causative agent. These HPV related tumour have a clinico-pathological profile that is quite divergent from conventional non-HPV related tumours. Association of HPV in oral cancers has not been explored in north-east India.

Materials and Methods: Thirty-one patients with oral cavity squamous cell carcinoma (OSCC) on treatment from October 2010 to January 2013 were included in the study. Patients who received neo-adjuvant chemotherapy were excluded. HPV 16/18 DNA was evaluated using Chromogenic in-situ Hybridization (CISH). Presence of nuclear signals was taken as positive HPV expression. p16 was evaluated using immunohistochemistry and was considered positive if ≥ 80% of the tumour cells showed strong and diffuse nuclear/cytoplasmic immunostaining. The results were analysed using Fisher exact test and confidence interval was calculated where required.

Results: The study group age ranged from 30 to 80 years (median age- 54.2 years). The most common site was gum, with well differentiated squamous cell carcinoma being the most common histology. HPV 16/18 DNA was positive in 29% (95% CI: 13.03% - 44.97%) cases and had a clear tendency towards statistical significance with non-smoker cases (p=0.05), lymph node metastasis (p=0.05) and a significant correlation with p16 overexpression (p=0.04). There was no significant correlation with other clinico-pathological parameters.

Conclusion: HPV 16/18 is associated with OSCC, commonly seen among non-smokers and may be related to nodal metastasis. So, HPV may be used as a prognostic factor in OSCC and p16 may be considered as a surrogate marker for HPV.

Keywords: CISH, HPV, Oral carcinoma

INTRODUCTION

The world has witnessed a steady increase in the incidence of global cancer burden in the last few decades. This is especially true for the developing countries; largely because of the growth in aged population along with an increase in cancer-causing behaviours. Globally around 263,900 new cases and 128,000 deaths from oral cavity cancer were reported in 2008. The highest oral cavity cancer rates were found in Melanesia, South-Central Asia and parts of Europe. Africa, Central America, and Eastern Asia reported the lowest oral cancer cases for both males and females [1].

In India, HNSCC accounts for 30-40% cancers at all sites, out of which 8-12% are oral cancers. In north east India, the prevalence of HNSCC is 54.48%. Among the HNSCC reported from north east India, oral cavity (16.28%) is the third most common site and it is preceded by oropharyngeal and esophageal cancer [2,3].

Many factors that are implicated for its causation include consumption of tobacco and betel nut in its various forms, alcohol, lack of awareness and viruses like HPV and Epstein Barr Virus (EBV) [2,4]. Molecular evidence indicates a relationship between high-risk HPV and the pathogenesis of oral cancers [5]. The IARC Multicenter Study estimated that 18% of oral and oropharyngeal cancers worldwide are HPV associated [6]. HPV is a DNA tumour virus with oncogenic potential. Out of the 130 subtypes identified, HPV 16 and 18 are high risk strains most closely related to carcinomas [7]. HPV *E6 & E7* oncogenes produce oncoproteins that are responsible for the degradation of p53 and Rb leading to dysregulation of the cell cycle.

P16 is a cellular protein involved in cell cycle regulation. It is a cyclin-dependent kinase 4 (CDK4) inhibitor, and is integral to Rb mediated regulation of G1-S phase of the cell cycle. p16 is expressed at a very low level in normal cell as Rb inhibits transcription of p16 [8]. The silencing of p16 by promoter methylation is commonly observed in HNSCC [9]. However, in HPV related tumours, functional inactivation of Rb by HPV E7 oncogene results in accumulation of p16 protein [8]. HPV positive tumours are highly correlated with p16 overexpression and it is considered to be a surrogate marker of the viral infection. In recent years, much attention has been given to the prognostic role of HPV status in HNSCC. Various studies have reported conflicting results. Some investigators have demonstrated no association between HPV and survival while others have confirmed a more favourable prognosis for patients with HPV positive tumours [5,10-12]. The variable outcomes are likely due to the heterogeneity of the patient populations, treatments received and HPV detection methods used [2].

The increasing number of HNSCC cases in North East India, especially among the younger age group, is a major cause of concern, as it is associated with high morbidity and mortality in a sizeable population [2,3]. Apart from the traditional risk factors like tobacco use, betel quid chewing and alcohol consumption, which is quite rampant in North East India, the HPV infection status and its association with the expression of the molecular marker p16 have not been investigated so far. This study was undertaken to answer this issue.

MATERIALS AND METHODS

Cases

Patients who were diagnosed with OSCC at North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences, Shillong, who agreed to receive surgical treatment at the Department of Otorhinolaryngology and for whom complete clinical details with follow up data was available were included in the study. Patients who had received any form of neoadjuvant therapy and those with history of concurrent or previous cancer in other organs were excluded from the study. From October 2010 to January 2013, 31 such cases were identified. Since the prevalence of HPV infection in OSCC in this part of India is not known, so work done by other authors at other regions of the world were consulted and the minimum sample size necessary to obtain result suitable for statistical analysis was estimated, keeping in mind the financial concern pertaining to the study. Institute Ethical Committee clearance was obtained before the study was undertaken. Patient history was collected prior to the beginning of treatment in all the cases and this included the age, gender, clinical, dietary and social habits. Patients were stratified into betel nut, alcohol or cigarette users or non-users. The users were the patients who consumed betel nut, alcohol or cigarette for a minimum period of five years and were current users or stopped the habit six months prior to the onset of the disease, all other patients were non-users. Postsurgery, the tissue diagnosis was confirmed by histopathological examination of biopsies obtained from the oral cavity. TNM staging was done for all the cases and tumours were segregated into early (stage I and stage II) and advanced stage (stage III and stage IV) [13].

HPV Chromogenic in-situ hybridization (CISH)

Formalin fixed and paraffin-embedded (FFPE) sections were evaluated for HPV using CISH (Zytovision, Germany). Sections of 5 μm thickness were taken on poly L-lysine coated slides, dewaxed in xylene, rehydrated in graded alcohol and incubated in 3% H_2O_2 and washed. Pepsin digestion was done for 10-20 minutes at 37°C in a humidity chamber, followed by heat pre-treatment in EDTA at 95° C for 15 minutes. A 10 μl of HPV 16/18 probes were pipetted onto each sample and were covered with a cover slip and edges sealed with rubber cement. The samples were then denatured at 75°C for 5 minutes on a hot plate and then transferred to a humidity chamber and hybridized at 37°C for 60 minutes. Cover slips were removed, followed by serial application of Mouse-anti-DIG, anti-Mouse-HRP-polymer and Diaminobenzidine chromogen (DAB). Any brown coloured dot like signal from the nucleus of the cell was considered positive. ZytoFast (+) DNA probe and known HPV 16/18 positive cervical carcinoma cases were taken as control.

p16 Immunohistochemistry (IHC)

p16 was evaluated using monoclonal antibody raised against p16 (Biogenex, CA, USA). Sections of 5 μm were deparaffinised using xylene and rehydrated with graded alcohol. Antigen epitopes were unmasked in 10 mmol/L citrate buffer pH 6.0. Microwave antigen retrieval (5 min, 450 W; 5 min, 600 W) was done. Primary antibody was added and incubated in humidity chamber at room temperature for 120 mins. HRP polymer was subsequently added and slides were incubated for 30 minutes. DAB chromogen was used for visualisation of the reaction. p16 was considered to be over-expressed if ≥80% of the tumour cells showed strong and diffuse nuclear or cytoplasmic immunostaining.

STATISTICAL ANALYSIS

HPV-positive and HPV-negative tumours were compared by the Fisher exact test.

RESULTS

Over the 28 months study period, 31 patients who had undergone resection for OSCC were tested for presence of HPV 16/18 DNA.

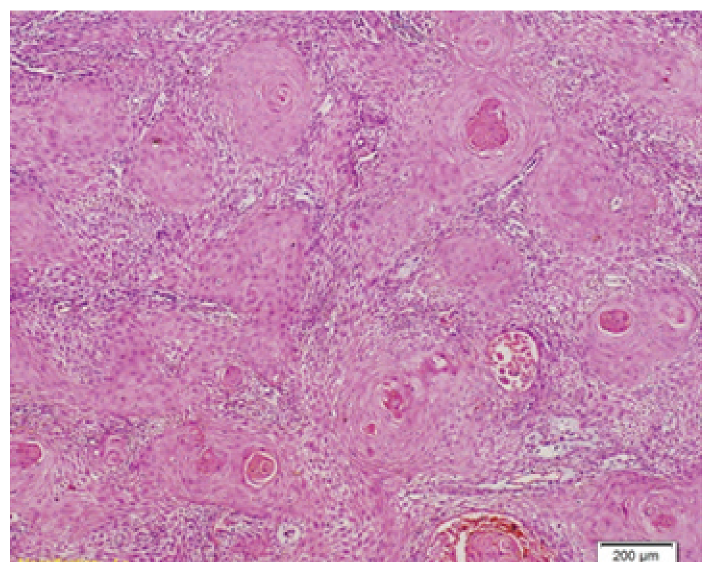
The clinical and pathological findings are summarized in [Table/Fig-1]. Patients age ranged from 30 to 80 years (mean, 54.2 years). The primary anatomical site affected by OSCC in our study was alveolus (n=13, 41.9%), buccal mucosa (n=8, 25.8%), tongue, lip, base of tongue (n=3, 9.7% each) and floor of mouth (n=1, 3.2%).

Grossly, the most common pattern observed was a proliferative growth pattern which constituted 52% (n= 16) of the cases. Majority of the cases were well differentiated squamous cell carcinoma on histopathological examination [55% (n=17)] [Table/Fig-2]. TNM staging was performed in all the cases. Twenty nine precent (n=9) cases were in early stage (stage I and II) and 71% (n=22) cases were advanced stage (stage III and IV) tumours.

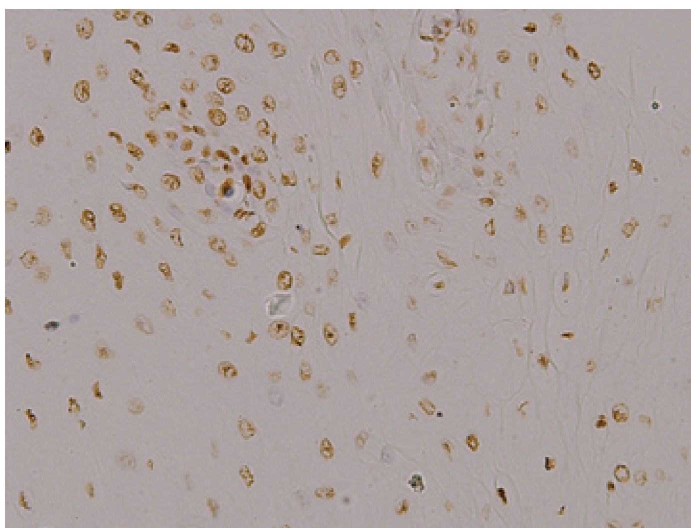
Twenty nine percent cases (95% CI: 13.03% - 44.97%) showed presence of HPV 16/18 DNA [Table/Fig-3] and 45% (n=14) of the total cases showed p16 overexpression [Table/Fig-4]. HPV related tumours were more common among non-smokers. When the

Patient or tumor Characteristic		Total (n=31)	HPV 16/18 Positive (n=9)	HPV 16/18 Negative (n=22)	P -Value
Sex	Male	16 (51.6%)	4 (44.4%)	12 (54.5%)	0.70
	Female	15 (48.4%)	5 (55.6%)	10 (45.5%)	
Age	≤50 years	13 (41.9%)	3 (33.3%)	10 (45.5%)	0.69
	>50 years	18 (58.1%)	6 (66.7%)	12 (54.5%)	
Betel nut	User	31 (100%)	9 (100%)	22 (100%)	—
	Non user	0 (0%)	0 (0%)	0 (0%)	
Tobacco	User	27 (87.1%)	7 (77.8%)	20 (90.9%)	0.55
	Non user	4 (12.9%)	2 (22.2%)	2 (9.1%)	
Smoking	User	19 (61.3%)	3 (33.3%)	16 (72.7%)	0.05
	Non user	12 (38.7%)	6 (66.7%)	6 (27.3%)	
Alcohol	User	9 (29%)	2 (22.2%)	7 (31.8%)	0.68
	Non user	22 (71%)	7 (77.8%)	15 (68.2%)	
Size	< 2 cm	1 (3.2%)	1 (11.1%)	0 (0%)	0.28
	2-4 cm	19 (61.3%)	5 (55.6%)	14 (63.6%)	
	> 4cm	11 (35.5%)	3 (33.3%)	8 (36.4%)	
Lymph Node	Metastasis present	12 (38.7%)	6 (66.7%)	6 (27.3%)	0.05
	Metastasis absent	19 (61.3%)	3 (33.3%)	16 (72.7%)	
p16 status	Over-expression	14 (45.2%)	7 (77.8%)	7 (31.8%)	0.04
	Normal/under-expression	17 (54.8%)	2 (22.2%)	15 (68.2%)	

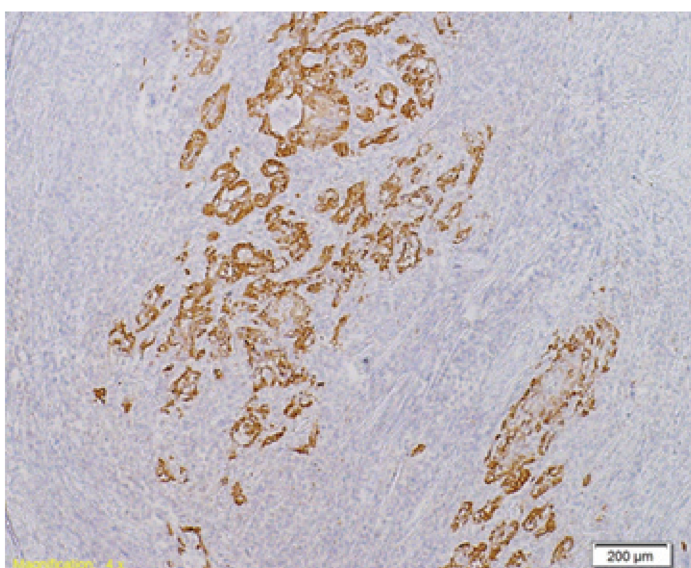
[Table/Fig-1]: Patient and tumour characteristic and p16 overexpression status in HPV 16/18 related oral cavity squamous cell carcinoma



[Table/Fig-2]: A case of well differentiated squamous cell carcinoma in the buccal mucosa (Haematoxylin & eosin stain, 100x)



[Table/Fig-3]: Human papilloma virus type 16/18 DNA positivity by Chromogenic in situ hybridisation. Brown dot like positive signal is seen in the malignant cells (in situ hybridisation, 200x)



[Table/Fig-4]: A case of oral cavity squamous cell carcinoma showing p16 overexpression. (immunohistochemistry, 100x)

cases of OSCC were stratified in accordance with the smoking habits, 66.7% (n=6) of the HPV 16/18 positive cases were non-smokers, compared to 33.3% (n=3) smokers, and a statistically significant result (p=0.05) was observed in this regard. Lymph node involvement was also found to be high in HPV 16/18 positive cases with 66.7% (n=6) cases showing lymph node metastasis compared to 27.3% (n=6) HPV 16/18 negative cases, the result being statistically significant (p= 0.05).

A statistically significant relation was seen between HPV positivity and p16 overexpression (p= 0.04). There was a 77.8% correlation rate between HPV 16/18 status and p16 positivity. The discrepant cases (i.e. HPV 16/18 negative by CISH but p16 positive by IHC) was probably because of non-16/18 type of HPV, warranting a more extended panel of in-situ hybridization (ISH) HPV probes [14].

DISCUSSION

The occurrence of OSCC has traditionally been linked to high degree of consumption of tobacco and betel nut in various forms. Similar trend are seen in north eastern India, especially with regard to betel nut chewing. There are two probable reasons that may explain why oral cavity is at high risk for development of carcinoma due to above mentioned risk factors. Firstly, carcinogens mixed with saliva, pool in the floor of the mouth, and constantly bathe these sites leading to constant carcinogen exposure; secondly, the thinner layer of stratified squamous epithelium at these regions of the oral cavity provides less protection against the carcinogens [15].

Recently, there has been a change in the risk factors and demographic profile of OSCC. Prevalence of HPV-dependent OSCC has been constantly increasing. Epidemiological data from the United States and Europe confirm that high-risk HPV of subtype 16 is detectable in oropharynx and the oral cavity [16]. Meta-analysis data showed that the average global prevalence of HPV in oral cancer ranged from 23.5% to 34.5% [17,18]. Our study showed HPV positivity in 29% of the cases clearly establishing HPV as an aetiological agent in OSCC. According to literature, sexual practices are the most probable mode of transmission of the virus to the oral cavity, compounded by alcohol and tobacco intake. Immune compromised state further aggravates the risk of transmission [19]. There are some proposed mechanisms of oncogenesis for HPV related OSCC. HPV viral genome can integrate into the cellular DNA. The early genes E6 and E7 of the HPV genome can be expressed by host cells. The E6 gene is related to the cellular E6-associated protein, which can degrade the tumour suppressor p53 protein, thereby causing tumours. E7 can promote the proteosomal degradation of pRb. These genes can promote malignant transformation of the host cell [20,21]. Bishop el al., in their in-situ hybridisation based study conducted on 282 HNSCC found that 17% of the tumours were associated with HPV and these cases showed significant correlation with p16 expression and concluded that their technique was technically more feasible for routine testing in clinical laboratory [22]. Schache et al., in their article discussed that persistent oncogene expression was required for initiation and maintenance of HPV driven carcinoma. Due to RNA instability, quantitative reverse transcriptase polymerase chain reaction, which is the gold standard in HPV detection, requires analysis of fresh-frozen tissue and specialist research laboratory techniques, which limits its use in routine clinical diagnostics. They described the use of a novel CISH based diagnostic algorithm using HPV RNA scope kit in oropharyngeal squamous cell carcinoma. The test was conducted on FFPE sections. The authors reported that the test had a sensitivity of 97%, specificity of 93%, positive and negative predictive values of 91 and 98%, respectively; against the reference test in detecting HPV infection. The authors highlighted the potential of this test to be used as a routine clinical standard for assigning diagnosis to HPV related carcinomas [23].

We found that HPV related OSCC was more common among non-smokers and approached acceptable levels of statistical significance (p=0.05). No such association was found with respect to betel quid and alcohol. Association of HPV related OSCC among non-smokers indicates that HPV might be an independent risk factor for oral carcinogenesis, acting through pathways that are different from those of chemical carcinogens [24]. The discrepant result with respect to alcohol consumption may be explained on the basis that HPV infected mucosal epithelium has greater susceptibility to subsequent chemical carcinogen exposure. The nature of these chemical carcinogens varies, based on the demographic, social and dietary habits of the people [10].

There was a strong tendency towards statistical significance between HPV 16/18 DNA positive tumours and lymph node metastasis in our study. Similar results were reported in other studies [9,10,25]. The higher incidence of lymph node metastasis in HPV related OSCC may be due to alteration in the function of the tumour suppressor genes following viral integration into host genome [10].

In the present study, significant correlation was seen between HPV positivity and p16 overexpression (p= 0.04), which was in concordance with the findings of other studies [14,26,27]. Binding of the E7 oncoprotein to the Rb protein leads to Rb protein degradation, presumably due to the compensatory overexpression of both cytoplasmic and nuclear p16 protein in HPV-infected tumour cells. This explains the up-regulation of p16 gene product in HPV related OSCC [28]. However, 22% HPV positive cases were not associated with p16 overexpression. These cases can be explained by the following: (1) HPV might have caused inactivation of p16 and

phosphorylation of Rb [2]. Intake of betel quid, smoking and alcohol might have caused the abnormal p16 function [29].

Compared to HPV negative tumours, in spite of higher rate of lymph node metastasis, studies have shown better overall survival, disease specific survival, and loco-regional control for HPV positive tumours [5,30,31]. Our cases were followed up for a median period of 18 months and showed similar results. Our study was limited by a small sample size and inadequate/short follow-up period, so definite conclusion on survival could not be made. A follow up of five years is warranted to bring out the real difference in survival between HPV related OSCC and non HPV related OSCC. The confounding effect of chemical carcinogens, especially betel nut and tobacco, delay in consultation, and higher tumour stage at the time of presentation might alter the survival data on long term follow up of patients in this region.

CONCLUSION

Oral carcinogenesis is a multi-factorial process involving socioeconomic, environmental and microbial factors leading to multistep changes. We observed that HPV infection was associated with significant number of cases and was found to be an independent risk factor as well as prognostic factor for OSCC. This might be useful for individualization of the therapeutic strategies in HPV related tumours. p16 is a surrogate marker for the virus and is technically less challenging to perform and hence can be utilised for determining the viral status.

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