RESEARCH COMMUNICATION

Role of Human Papilloma Virus in Oral Tongue Squamous Cell Carcinoma

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Abstract

Background: Human papilloma virus (HPV) is an important risk factor for head and neck cancer, specifically oropharyngeal cancer, but its association with oral tongue squamous cell carcinoma (SCC) is uncertain. The objectives were to determine the HPV16 prevalence in oral tongue SCCs, its integration status and to correlate the expression of oncogenic proteins with targets. Methods: In this case-control study with oral tongue SCC cases (n=60) and normal oral mucosa (n=46), HPV positivity was determined by polymerase chain reaction (PCR) using consensus and HPV 16 type specific primers and p16 immunohistochemistry (IHC). The viral integration status was determined with primers specific to the E2 gene and *in situ* hybridization (ISH). Immunohistochemical analysis of HPV oncogenic proteins (E6, E7) and their target proteins (p53, pRb, cyclinD1, p16, Notch-1, EGFR) proteins was carried out in HPV positive cases. The data was analyzed with SPSS software (v 11.0). Survival analysis was carried out by the Kaplan-Meier method. Results: HPV16 was detected in 48% (n=29) of the cases and none of the controls by PCR assay (p<0.001) while p16 IHC, as a surrogate HPV marker, detected 33% (n=18) of the cases; 18% (n=10) were detected by both the methods. Integration was observed in 83% (n=24) by E2-PCR and 67% (n=18) by ISH. The E6-p53 pathway was active in 33% of the cases; E7-pRb in 52% and both in 11%. HPV positivity was associated with well-differentiated cancers (p=0.041) and low recurrence rate (p=0.014). Conclusion: Our study confirms a positive correlation of HPV infection with oral tongue cancer.

Keywords: Tongue SCC - HPV - PCR - IHC - carcinogenesis

Asian Pacific J Cancer Prev, **12**, 889-896

Introduction

Tobacco and alcohol have been attributed as major risk factors for oral squamous cell carcinoma. With the decrease in prevalence of these habits, a parallel reduction in the incidence of head and neck cancer is observed in high risk countries, specifically in sub-sites having strong association with tobacco abuse–larynx and gingivo-buccal carcinoma (Elango 2006). Despite this reduction in overall incidence of head and neck cancer, there is an increase in incidence in certain sub sites such as oropharynx (Shiboski 2005), tongue (Elango 2006) and oral cancer in young adults (Macfarlane 1987). An increasing number of these patients that develop oral and oropharyngeal cancers are without any known local risk factors; implicating etiologic factors other than tobacco and alcohol as its cause. During the past three decades, data supporting human papilloma virus (HPV) as a causative agent in the development and progression of head and neck cancer, particularly that of oropharynx has accumulated. The overall HPV incidence varies depending on tumor location (McKaig 1998; Gillison 2001), the technique employed in the detection of the virus and geographic location of the patients. While two high-risk HPV types 16 and 18 are closely associated with cervical cancer, HPV16 is predominant in head and neck cancers. However, association between oral tongue cancer and HPV has not yet been established.

The primary objective of this study was to determine the prevalence of HPV16 in oral tongue cancers. The secondary objectives were to i) identify the HPV integration status, ii) correlate HPV positivity with the expression of HPV oncogenic proteins (E6 and E7) and their target cell cycle regulatory proteins (p53, pRb, p16, cyclinD1, Notch-1, EGFR)

Materials and Methods

Cases and Controls

Patients diagnosed with histologically confirmed squamous cell carcinoma of the oral tongue were recruited

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for the study. Tumor staging was assessed according to the American Joint Committee on Cancer staging criteria (UICC 6th ed., 2002) and histological grading was performed following the WHO criteria for oral squamous cell carcinoma. The tissue samples of 60 consecutive patients diagnosed with oral tongue cancer accrued from 2004 to 2007 were taken from the repository at the Head and Neck Department of the tertiary care comprehensive cancer centre. The cases were assigned to sub categories based on their characteristics (age, sex and risk factors) and matched controls were accrued. Normal oral mucosa of subjects without a history of cancer formed the controls. One hundred and twenty control samples were collected; out of which 46 subjects frequency- matched to the cases by 5-year age categories, sex and the prevalence of risk factors (Table 1). The samples from cases and controls were collected after obtaining institutional review board approval and with their informed consent.

Demographic details, risk factors, tumor characteristics and disease status were obtained from the patient records. Presence of risk factors was defined as use of tobacco, pan chewing or consumption of alcohol at least five days per week for a minimum period of two years. Overall survival and disease-free survival were measured in months from the date of diagnosis until death or until the patient was last known to be alive and until recurrence respectively. Dates of death or dates last known to be alive were obtained from the medical records and the department follow-up register.

HPV Detection

The presence of HPV in tissue was determined by polymerase chain reaction (PCR) and p16 expression by Immunohistochemistry (IHC). The integration of HPV16 in host genome was confirmed by in situ hybridization (ISH) and the disruption of the early gene E2 by PCR. Briefly, DNA was isolated from the tissues by phenol chloroform method and the quality of DNA was determined by amplification of housekeeping gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase). PCR was carried out with the HPV consensus primers MY11/09(Karlsen 1996). The samples negative for this primer were tested with two other consensus primer sets; GP5+/6+, CPI/II (Karlsen 1996) to exclude false negatives. HPV16 specific primers (HPV16 L1 and E6) (Karlsen 1996; May 1996; Gallo 2003) were then used to identify the virus type. The integration of the HPV DNA into the host DNA was assessed by PCR using E2-specific primers(Gallo 2003). The details of primers are given in Table 2. The PCR reaction conditions were as follows: initial denaturation at 94˚C for 5 minutes, 40 cycles of denaturation at 94˚C for 1 minute, annealing at 55˚C–61.7˚C for 1 minute; extension at 72˚C for 1 minute followed by a final extension at 72˚C for 7 minutes. Representative amplicons obtained with each primer pair were subjected to DNA sequencing by Sanger's method(Sanger 1977) and compared with a standard sequence (GenBank K02718/HPV16R) to confirm the specificity of the amplified product.

p16 over-expression, which is considered as a surrogate marker for HPV16, was determined by immunohistochemistry using p16 mouse monoclonal antibody (cat # sc-65224, Santa Cruz, CA). IHC was carried out according to standard protocols in 55 cases for whom the tissue blocks were available. The sections were deparaffinized, rehydrated and incubated overnight with the primary antibody at 4°C. The sections were visualized using the high sensitivity peroxidase-DAB system (Dako REAL™ ENVISION™ Detection System, Denmark).

Table 1 Characteristics of Cases and Controls

Ch aracteristic	Cases	Controls	p
Sex (%)			
male	41(68%)	35(76%)	0.508
female	19(32%)	11(24%)	
Mean age in years (SD*)			
male	55.5 (11.9)	54.7 (13.6)	0.313
female	54.1 (16.1)	44.9 (12.4)	
Risk factors (%)			
present	30(50%)	25(54%)	0.804
absent	30(50%)	21(46%)	

*SD: Standard deviation

Table 2. List of Primers for HPV PCR Analysis

Name	5* 3* Nucleotide Sequence	Target/size (bp)
GAPDH-F	TCACCAGGGCTGCTTTTAACTC	GAPDH/150
GAPDH-R	ATGACAAGCTTCCCGTTCTCAG	GAPDH
MY 11	GCMCAGGGWCATAAYAATGG	HPV L1/450
MY09	CGTCCMARRGGAWACTGATC	HPV L1
GP5+	TTTGTTACTGTGGTAGATACTAC	HPV L1/150
GP6+	GAAAAATAAACTGTAAATCATATT	HPV L1
CP I	TTATCWTATGCCCAYTGTACCAT	HPV E1/188
CP II	ATGTTAATWSAGCCWCCAAAATT	HPV E1
HPV16 F	TGCTAGTGCTTATGCAGCAA	HPV16 L1/152
HPV16 R	ATTTACTGCAACATTGGTAC	HPV16 L1
E6 F	AAGGGCGTAACCGAAATCGGT	E6/209
E6 R	TTGGTCACGTTGCCATTCAC	E6
E2 F	CTTGGGCACCGAAGAAACAC	E2/351
E2 R	TTGGTCACGTTGCCATTCAC	E2

In situ Hybridization

The samples positive by PCR were subjected to Catalyzed signal Amplified colorimetric in situ hybridization (CSAC-ISH). The 30 mer biotinylated HPV16 probe specific to the L1 region (GCAAACCACCTATAGGGGAACACTGGGGCA) (May 1996) was used to confirm the presence of HPV16 in the tumor cell. ISH was carried out using standard protocols; briefly, the section were deparaffinized, rehydrated and processed for hybridization. The sections were overlaid with the probe cocktail (40% deionised formamide, 0.25% BLOTTO, 20X SSC, 1mg/ml sheared salmon sperm DNA, 10mM DTT, 10% dextran sulphate), denatured and incubated at 42°C overnight. The sections were then subjected to stringent washing protocols and the signals amplified using the Tyramide Signal Amplification (TSA) plus amplification TSA™ DNP (AP) System (NEL746A001KT, Perkin Elmer, MA, USA). Dark blue punctate or diffuse nuclear staining was regarded as positive signals for HPV. The episomal virus leads to diffuse staining, while integration of viral DNA into host cell DNA gives punctate signals. ISH analysis was carried out in the 27 out of 29 patients' positive for HPV-16, for whom the tissue blocks were available.

Table 3. Details of Antibodies used

Antibody	Dilution	Incubation	Staining pattern	Positive control	No*
E6	1:25	1hour 37°C	N,C	CC	sc-460
E7	1:100	Overnight 4°C	N,C	CC	sc-58661
p53	1:250	1hour 37°C	N	BC	Neo Ab-3
pRb	1:25	Overnight 4°C	N	BC	Novo
p16	1:50	Overnight 4°C	N,C	CC	sc-65224
CyclinD1	1:50	1hour 37°C	N	CC	Neo SP4
Notch-1	1:50	1hour 37°C	C,N,M	BC	Neo Ab-1
EGFR	1:50	Overnight 4°C	M	HNSCC	sc-03

*Catalogue no; N, nuclear; C, cytoplasmic; M, membranous; CC, cervical cancer; BC, breast cancer; sc, Santa Cruz, CA, USA; Neo, Neomarkers, CA, USA; Novo, Novocastro NCL-RB-358

Table 4 Prevalence of HPV by PCR and p16 IHC*

		PCR Positive No. of cases (%)	PCR Negative No. of cases (%)	TOTAL No. of cases (%)
p16 IHC	Positive	10(18%)	8(15%)	18(33%)
	Negative	19(35%)	18(33%)	37(67%)
	TOTAL	29(53%)	26(47%)	55

*IHC: Immunohistochemistry; Percentages represent positivity in 55 patients for whom the tissue blocks were available

Table 5. Correlation of HPV Status with the Characteristics of Cases

Characteristics		Total (No. 60)	HPV+ve (No. 29)	HPV-ve (No. 31)	p value
Gender	male	41 (68)	22 (76)	19 (61)	0.349
	female	19 (32)	7 (24)	12 (39)	
Age	≤45 years	16 (27)	8 (28)	8 (26)	0.891
	>45 years	44 (73)	21 (72)	23 (74)	
Risk factors	present	30 (50)	17 (59)	13 (42)	0.301
	absent	30 (50)	12 (41)	18 (58)	
Grade	WD	34 (57)	21 (72)	13 (42)	0.041
	MD	22 (37)	6 (21)	16 (52)	
	PD	4 (6)	2 (7)	2 (6)	
Stage	I	19 (32)	10 (34)	9 (29)	0.975
	II	11 (18)	5 (17)	6 (19)	
	III	13 (22)	6 (21)	7 (23)	
	IV	17 (28)	8 (28)	9 (29)	
Recurrence		12 (20)	2 (7)	10 (32)	0.014

The percentages are given in parentheses. WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated

IHC of HPV oncogenic proteins and the cell cycle proteins

As evidenced by literature, there are two major pathways (E6-p53 & E7-pRb) involved in HPV mediated carcinogenesis. The expression of the oncogenic proteins E6 & E7 and their target proteins- p53, pRb, p16 and cyclin D1 was analyzed by immunohistochemistry. IHC was carried out in the 27 out of 29 patients' positive for HPV-16, for whom the tissue blocks were available. The expression of Notch-1 and EGFR, the other known E6-E7 targets were also analyzed in these cases. IHC was performed as mentioned previously. The details of the antibodies and their working condition are given in Table 3.

Immunostaining for all antibodies was quantified by counting the cells exhibiting positive staining with

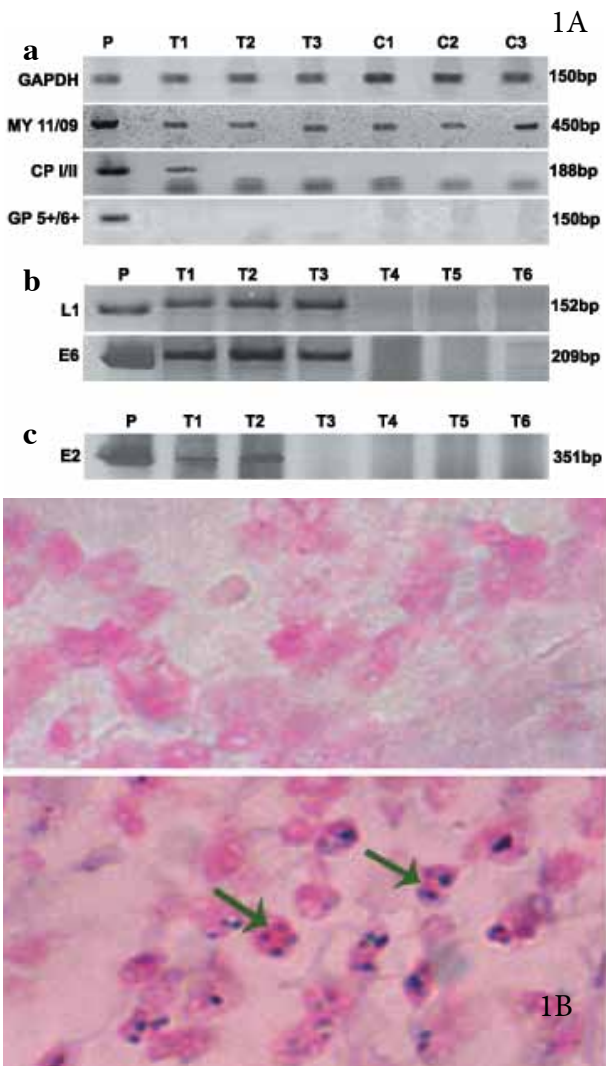

Figure 1 Detection of HPV. 1A Polymerase chain reaction in representative tumor and control DNA samples. (a) Amplicons obtained by PCR using primers specific for GAPDH and HPV consensus primers (MY11/09, GP5+/6+, CPI/II). Lane P represents HPV positive control, lanes T1, T2, T3 the patient samples and lanes C1, C2, C3 the control samples. (b) Amplification of the samples positive for consensus primers by HPV 16 specific primers for L1 and E6 genes. (c) Amplification of the E2 gene in the samples positive for HPV 16. In Fig 1Ab & 1Ac, lane P represents the HPV16 positive control and the lanes T1-T6 the patient samples. 1B Representative Sample of in-situ hybridization in a HPV case. Original magnification x 400 (b) Green arrows indicate the punctate signals obtained after in situ hybridization demonstrating the integrated HPV 16 virus. (a) represents the negative control.

a given antibody in 10 randomly selected high-power fields (40X) and the results were expressed as percentages of all epithelial cells in those areas (minimum of 2,000 cells). Two independent observers who were blinded to the outcome performed cell counting. E6 and E7 expression was specified based on the presence or absence of immunoreactivity. In the case of the other proteins, it was considered sig nificant when characteristic nuclear/cellular/membrane immunoreactivity was seen in at least 10% of the tumor cells. In addition to this, an expression index was also created. The protein expression was classified into four categories; grade 1: less than 10% positive cells (insignificant); grade 2:11–30% positive

cells (mild expression), grade 3: 31–60% positive cells (moderate expression), and grade 4: more than 61% positive cells (intense expression).

Statistical analysis

Statistical analysis was carried out using SPSS, version 11.0. Chi-square test was employed to test the association of different variables with gender and HPV status. Independent sample 't' test was employed to compare the mean age of cases and controls; HPV positive and negative cases. Survival analysis was estimated by the Kaplan–Meier method and the significance of difference between curves was tested by the log-rank test.

Results

Details of study groups

The study was carried out with 60 cases and 46 controls. The patients' age ranged from 28 to 83 years (mean=55 years, SD=13.3), with 68% males and 32% females. Among the controls, 76% were males and 24% were females, the age ranged from 27 to 80 years (mean=52 years, SD=13.8). Twenty-five (54.3%) controls and 30 cases (50%) had at least one of the risk factors. The cases and controls were statistically comparable with respect to gender, mean age and the prevalence of risk factors. There was no significant difference between males and females with respect to staging and grading among the cancer cases.

Prevalence of HPV

PCR using the consensus primers revealed thirty patients (50%) and thirty-one (67%) subjects in the control group as positive for HPV infection (Figure1A (a)). The samples positive for HPV infection were tested for oncogenic HPV16; 29 cases (48.3%) (Figure1A (b)) and none of the controls were positive (p=0.001). Sequencing of representative amplicons obtained from each primer pair and analysis by Basic Local Alignment Search Tool (BLAST, NCBI) confirmed the specificity of the sequence. The use of p16 as a surrogate marker revealed 18/55 (33%) subjects as positive for HPV. Eighteen percent (10/55) were positive by both HPV16 PCR and p16 IHC (Table 4). p16 expression was found to be below the detection level among the controls. The use of multiple consensus primer sets in PCR was with the objective to avoid false negatives with regard to HPV infection in general. The cases positive for HPV infection by PCR were hence selected for HPV16 typing.

HPV Integration

The integration of the HPV into the host DNA was detected by PCR with E2-specific primers (Figure 1A(c)). If the virus is integrated, E2 oncogene will be disrupted; hence the presence of HPV infection with loss of E2 was considered indicative of integration. Twenty four out of twenty nine (83%) patients showed HPV integration by

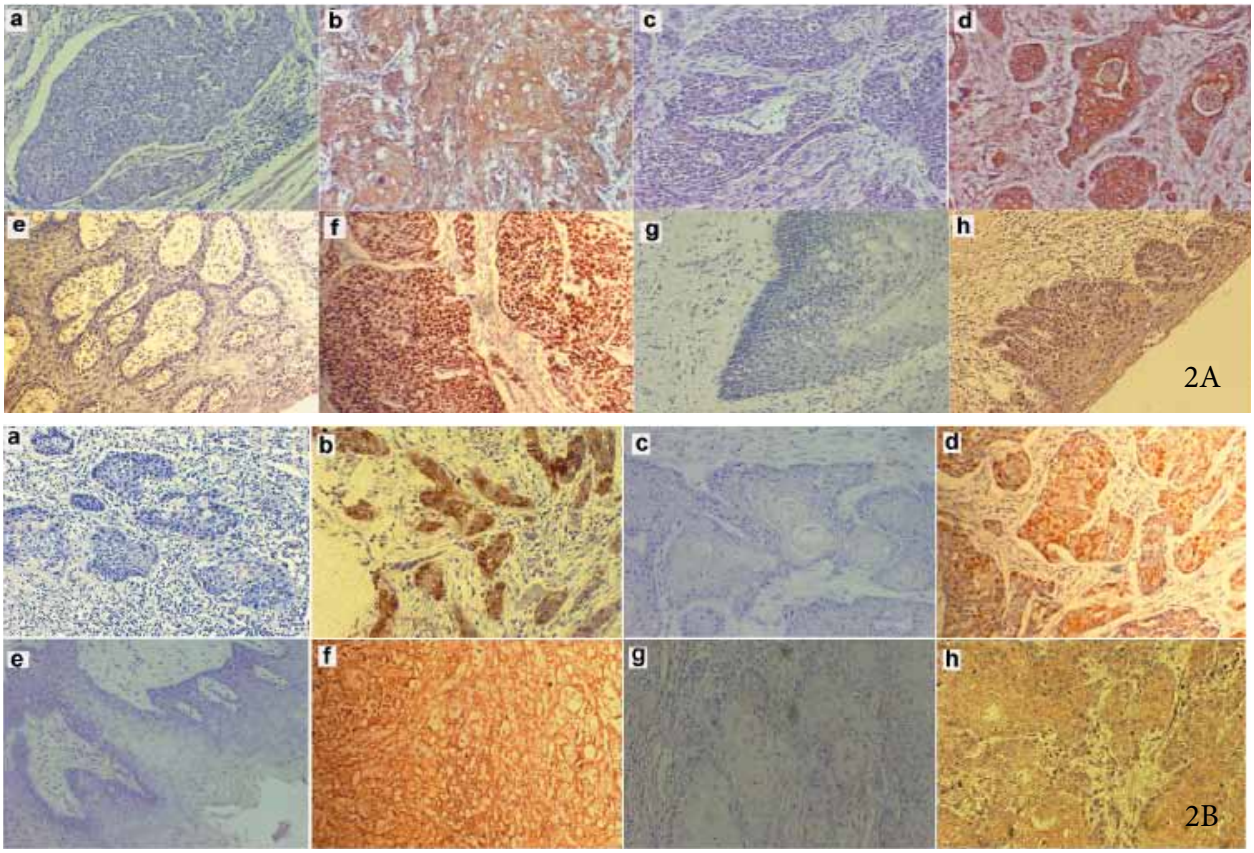

Figure 2. Representative Immunostaining of E6, E7, p53, pRb, p16, cyclinD1, EGFR and Notch-1 proteins in HPV 16 Positive Cases. Original magnification x100 A (b) and (d) Cytoplasmic expression of the HPV oncoprotein E6 and E7 in tongue cancer respectively. (f) and (h) Nuclear staining of tumor suppressor genes p53 and pRb. (a,c,e,g) represent the negative controls for E6, E7, p53, pRb respectively. B (b) Nuclear and cytoplasmic staining of p16 (d) nuclear staining of cyclin D1 (f) Membranous staining observed for EGFR (h) Cytoplasmic staining with Notch 1 (a,c,e,g) represent the negative controls for p16, Cyclin D1, EGFR and Notch 1 antibodies respectively

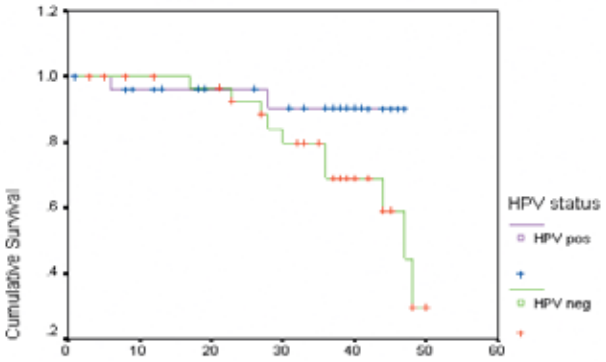

Figure 3. Disease-free Survival by HPV status. The green line is for HPV negative and the pink line for HPV positive patients (p=0.014)

E2 PCR. Further confirmation of HPV DNA integration in the tumor DNA was performed by catalyzed signal amplified colorimetric in situ hybridization (CSAC-ISH). Sixty-seven percent (18/27) of the PCR positive cases showed integration as revealed by punctate signals in the tumor nuclei (Figure1B(b)).

IHC analysis of HPV oncogenic proteins and the target cell cycle proteins

Our objective was to delineate the percentage of oral tongue cancer patients with HPV16 infection wherein the virus actually plays a role in carcinogenesis and the specific pathways (E6-p53, E7-pRb) it follows. The proteins assayed in this study are known to have aberrant expression in tongue cancers depending on the varied mechanisms of carcinogenesis involved. It was hence considered relevant to evaluate the expression of these proteins in only the HPV positive cases.

Expression frequency of the proteins

The expression of E6 protein was observed in 63% and E7 in 78% of the cases (Fig 2A (b) and Fig 2A (d)). Expression of p53 and pRb was observed in 56% and 11% of the cases respectively (Fig 2A(f) and Fig 2A(h)). A loss of p53 was observed in 44% of the cases as compared to pRb (89%). The cell cycle regulatory protein cyclin D1, downstream to pRb, was down-regulated in 67% of the patients. p16 over expression was observed in 37% (10/27) (Figure 2B(b)). Eighty-nine percent and 26% of the cases were positive for EGFR and Notch-1 respectively (Figure 2B(f)and 2B(h)).

Expression pattern of the E6-p53 pathway proteins

Nine cases (33%) were positive for E6 and had an accompanied loss of p53 indicating that the pathway might be active in this subset of HPV16 positive patients.

Expression pattern of the E7-pRb pathway proteins

Eighteen cases (67%) showed E7 expression with a concordant loss of pRb. Further analysis revealed a subset of 19% (5/27) of the cases with a profile of E7+/pRb-/p16+ suggesting an involvement of this pathway in the carcinogenic process. Fifty two percent (14/27) of the cases which were E7+/pRb- showed either p16 over expression or cyclin D1 loss.

Eighty nine percent of the HPV positive cases showed either E6 or E7 expression and 52% showed expression of both the proteins. A combined analysis revealed that eleven percent (3/27) had both the pathways active and 81% (22/27) had either of the pathways active.

Expression of Notch1: Eighteen cases (67%) showed down regulation of Notch 1 along with E6/E7 expression.

Survival outcome

The patients had a mean follow up of 24 months. The overall survival rate was 80% and the overall mean survival duration was 43 months (95% CI: 38-46). Twelve patients (20%) had recurrence, 5 in the primary site, 4 had nodal recurrence and 3 skeletal metastasis. There was no statistically significant difference in the survival rate among cases with respect to different clinical and pathologic variables. Also, there was no significant difference between the cases positive and negative for HPV16 infection (p=0.714).

The demographic and histopathologic characteristics of the HPV positive and negative patients were compared in order to document any major differences between the two groups. There was no significant difference with respect to gender, age group, presence of risk factors and staging. HPV positivity was more common in well-differentiated cancers (p=0.041) (Table 5). Disease recurrence was 7% (n=2) in HPV positive tumors as compared to 32 % (n=10) in the HPV negative tumors (p=0.014) (Figure 3).

Discussion

While there is reduction in the overall incidence of head and neck cancer in parallel with lowering of tobacco and alcohol consumption (Elango 2006), there are convincing epidemiologic studies demonstrating an increase in the incidence of carcinoma of tonsil and oral tongue (Shiboski 2005). These two head and neck cancer sub-sites have the least association with tobacco and alcohol abuse. HPV has been recently implicated as a causative agent for carcinoma of tonsil; however there is paucity of data analyzing the association of oral tongue cancer and HPV.

Systematic review of 60 studies involving 5026 head and neck cancers has shown 25.9% HPV positivity (Kreimer 2005), which is most pronounced in carcinoma of tonsil. In most of the studies investigating oral cavity, tongue cancers formed a subset of oral squamous cell cancers, rather than a separate sub-group. It is suggested that tonsillar crypts act as reservoirs for the virus and hence the relatively increased prevalence among tonsillar cancers (Syrjänen 2004; Hammarstedt 2006). Recently, it has also been observed that periodontal pockets may act as reservoirs for human papilloma virus (Hormia 2005). Tezal et al have shown the association between long-standing periodontitis and risk of tongue cancers (Tezal 2007).

The frequency of detection of HPV in tongue cancers ranged from 0-81 (Hönig 1992; Balaram 1995). The HPV detection rates vary depending on the assay employed and geographic location of the study population. The highest rate was detected by Balaram by Balaram et al (Balaram 1995) in his study on the prevalence of HPV

infection among betel quid chewers in India, wherein it was observed that 9 out of 11 subjects were positive for HPV infection. The sample size used in previous studies on tongue cancers ranged from 7 to 61, majority of the studies employing either PCR or in situ hybridization as the detection method (Kantola 2000; Koskinen 2003; Liselotte 2004; Xin-Hua Liang 2008). In our study, multiple PCR assays (consensus and HPV 16 specific primers) were carried out in order to avoid false negatives and the integration of the virus into the host genome, essential for its pathogenesis, was demonstrated by E2 oncogene disruption and ISH. It was observed that of the 48% of the tongue cancers were positive for HPV16, 83% showed integration by E2 PCR and 67% by ISH, suggesting the role of HPV in their carcinogenesis. The sensitivity of ISH was found to be at least 20-50 copies per cell, hence the difference in the positivity rate. A similar reduced positivity rate with ISH detection as compared to PCR has been reported in previous studies (Kim 1997). The higher HPV prevalence observed in this study is in concordance with the observation that HPV infection in oral SCC is more prominent in India (20-50%) as compared to other countries (Noureen M 2009).

p16 over-expression is highly correlated with HPV infection in cervical cancers speculating that it may be used as a surrogate to HPV DNA detection by routine methods (Klaes 2001). In our study, the HPV prevalence as detected by p16 IHC (33%) was less as compared to PCR (48%). Fifteen percent of the cases with p16 over expression were negative for HPV infection by PCR suggesting that other causes like non-HPV16 infection or activation of alternate pathways might be responsible for the increase in protein expression.

Mere detection of HPV may also not be indicative of its involvement in carcinogenesis. The viral integration and subsequent aberrant expression of the proteins involved in the respective pathways provide more conclusive evidence. In our study, the expression of E6 and E7 oncogenic proteins was observed in 63% and 78% of the cases positive by HPV16 PCR, respectively. A combination of PCR and immunohistochemical methods detected 81% (22/27) of the cases positive by PCR with active E6-p53 or E7-pRb pathway indicating HPV16 as responsible for the carcinogenic process in this subset of cases. The use of any single marker/detection method may not hence provide accurate information regarding the association of the virus with the disease.

There is a strong notion that HPV plays a role in carcinogenesis in subjects without established risk factors (Lindel 2001). Balaram et al has previously reported high incidence of HPV infection in tobacco chewers (Balaram 1995). Numerous studies have reported the additive effect of tobacco and alcohol consumption and HPV (Schwartz 1998; Smith 2004). Comparison of patients with and without risk factors in this study, did not show any statistically significant difference in the incidence of HPV16 infection (p=0.301) (Table.5). Hence, HPV may act as an independent risk factor or with other established risk factors like tobacco and/or alcohol to induce carcinogenesis.

HPV positive tumors may have different clinical and biologic behavior, with improved overall survival and favorable prognosis (Licitra 2006). They have been reported to show better response to chemotherapy and radiotherapy (Fakhry 2008). Previous studies reveal that HPV positive cancers were more common in the younger age group (Mellin 2000; Strome 2002), non smokers (Gillison 2004), associated with advanced TNM stage and poorly differentiated tumors (Haraf 1996; Wilczynski 1998; El-Mofty 2003). In our study it was found that cases positive for HPV16 had a lower recurrence rate as compared to their negative counterparts, pointing out to an association between HPV16 infection and a good prognosis. Fakhry et al reported a significant improvement in the outcome to chemo-radiotherapy among laryngo-pharyngeal patients positive for HPV infection (Fakhry 2008). In the present study, where surgery was used as a primary modality of treatment, a similar beneficial effect of HPV infection was observed, suggesting that improvement in loco-regional disease control of HPV positive cases is independent of choice of treatment modality.

In this study, HPV infection appears to be associated with well differentiated tongue squamous cell carcinoma. This is in contrast to oropharyngeal carcinoma which are generally poorly differentiated, where HPV infection is implicated as a major risk factor (Kies MS 2009). In HPV mediated cervical cancers, it is associated with both well differentiated and poorly differentiated cancers (Lo 2001; Pilch 2001). Majority of the cases accrued for this study, as is seen in the general oral tongue cancer patient population, have well differentiated squamous cell carcinoma (57%) while poorly differentiated constituted only 6% of the cases. From these findings, it is difficult to establish whether HPV is responsible for tumor differentiation.

HPV E6 protein mediates the degradation of p53 through the ubiquitin pathway. Nevertheless, tumors positive for HPV16 are also reported to have an active p53 protein (Gillison 2000; Wiest 2002; Balz 2003); the latter might be as a result of excess synthesis of p53 due to increased DNA damage. Pillai et al have shown that the aberrant expression of high risk HPV 16/18 E6 protein is a critical event in HPV carcinogenesis (Pillai 1999), which is further increased in the integrated virus due to a non-functional E2 gene (Schwarz 1985; Cullen 1991; Jeon 1995). In our study, concomitant E6 expression was observed with p53 degradation and expression in 33% and 18% of the cases respectively. The expression of E7 and the absence of pRb suggest the formation of a complex between the two proteins, leading to pRb degradation; thus implicating this pathway in HPV mediated carcinogenesis. In our study, immunohistochemical results revealed 33% of HPV positive patients with an active E6-p53 and 52% with E7-pRb pathway active. Both the pathways were active in 11% of the cases. Eighty one percent of the cases had either of the pathways active. For the rest of the HPV positive patients, different mechanisms might be involved in carcinogenesis. Cyclin D1 down-regulation is one of the known downstream effects of reduced pRb expression. In our study, it was observed that 44% showed cyclinD1 loss with E7 expression and pRb degradation. As is evident from these results, the E7-pRb pathway might have been the predominant pathway in the carcinogenesis among the

HPV infected cases in this study.

The present study showed a high percentage (67%) of HPV positive patients with down regulation of Notch-1 with E6/E7 expression. Notch1 is known to exert specific protective effects against HPV-induced transformation through suppression of E6/E7 expression (Talora, 2002), and its down-modulation indicates a causative role in this subset of patients. EGFR activation might be due to E5 mediated post translational mechanism (Pim, 1992; zur Hausen, 1996; Crusius, 1998) or due to HPV E6 and E7 activation (Conrad, 1994; Hwang, 1995). In our study it was observed that 89% of the HPV positive cases showed EGFR expression. Further investigations like immunoprecipitation studies should be carried out to better understand or confirm the molecular mechanisms involved.

In conclusion, the present study shows a positive correlation of HPV16 with oral tongue cancers. The expression studies indicate that both E6-p53 and E7-pRB pathways are involved in HPV mediated carcinogenesis, with E7-pRb pathway preponderance. This study also suggest improved loco-regional control in subjects with HPV positive tumors treated by surgery, as observed with chemo-radiotherapy in laryngo-pharyngeal cancers.

Acknowledgements

This project was supported by KSCSTE-SARD research grant: 031/RD AUG04/CSTE. The authors are thankful to Dr Radhakrishna Pillai, Director, Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram for providing us the HPV positive controls. The authors are also grateful to Dr K. Thangaraj, Scientist, Centre for Cellular & Molecular Biology (CCMB), Hyderabad, Ms Sindhu VG, Research Scholar, MSCC, Bangalore and Mr Muhil Vannan, Project Assistant, AIMS, Kochi for their help in sequencing analysis. The authors thank Ms Jeena, for her help in sectioning the blocks for IHC/ISH.

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