RESEARCH ARTICLE

Risk Stratification of Early Stage Oral Tongue Cancers Based on HPV Status and p16 Immunoexpression

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Abstract

Background: Recent epidemiological data have implicated human papilloma virus (HPV) infection in the pathogenesis of head and neck cancers, especially oropharyngeal cancers. Although, HPV has been detected in varied amounts in persons with oral dysplasia, leukoplakias and malignancies, its involvement in oral tongue carcinogenesis remains ambiguous. Materials and Methods: HPV DNA prevalence was assessed by PCR with formalin fixed paraffin embedded sections (n=167) of oral tongue squamous cell carcinoma patients and the physical status of the HPV16 DNA was assessed by qPCR. Immunohistochemistry was conducted for p16 evaluation. Results: We found the HPV prevalence in tongue cancers to be 51.2%, HPV 16 being present in 85.2% of the positive cases. A notable finding was a very poor concordance between HPV 16 DNA and p16 IHC findings (kappa<0.2). Further molecular classification of patients based on HPV16 DNA prevalence and p16 overexpression showed that patients with tumours showing p16 overexpression had increased hazard of death (HR=2.395; p=0.005) and disease recurrence (HR=2.581; p=0.002) irrespective of their HPV 16 DNA status. Conclusions: Our study has brought out several key facets which can potentially redefine our understanding of tongue cancer tumorigenesis. It has emphatically shown p16 overexpression to be a single important prognostic variable in defining a high risk group and depicting a poorer prognosis, thus highlighting the need for its routine assessment in tongue cancers. Another significant finding was a very poor concordance between p16 expression and HPV infection suggesting that p16 expression should possibly not be used as a surrogate marker for HPV infection in tongue cancers. Interestingly, the prognostic significance of p16 overexpression is different from that reported in oropharyngeal cancers. The mechanism of HPV independent p16 over expression in oral tongue cancers is possibly a distinct entity and needs to be further studied.

Keywords: Oral tongue cancer - HPV infection - p16 expression - PCR - immunohistochemistry - prognosis

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Introduction

Carcinoma of the oral tongue is one of the most common sub sites of oral cancer. Comparative worldwide studies have reported its incidence to be much higher in India (Moore et al., 2000; Mishra and Meherotra, 2014). In Chennai, India, oral tongue squamous cell carcinoma (OTSCC) represents 5% and 1.6% of incident cancers of all sites among males and females respectively as per the reports of population based cancer registry of the National Cancer Registry Program. The Age Adjusted Incidence Rate (AAR) of carcinoma tongue has shown an increasing trend over the past 25 years, from 3.6 in 1982-1983 to 5.7 in 2004-05 according to National Cancer Research Program (NCRP 2005). The trends in epidemiology of oral cancer in Asia in the past decade between 2000-2012 has shown that tongue is the most frequently affected site (Sreevidya Krishna Rao et al., 2013). The trends of head and neck cancers from Rural and urban India shows predilection for tongue cancers despite the decrease in the incidence of other oral cavity cancers. (Elango et al., 2006) Earlier reports have shown that tongue cancer has increased among the non-tobacco users, suggesting a review of the clinico- epidemiological factors and molecular changes in OTSCCs. (Mafi et al., 2012; Arvind Krishnamurthy et al., 2013; Bektas-Kayhan et al., 2014)

Human Papillomavirus (HPV), an epitheliotropic DNA virus, is known to be an etiologic agent in the tumorigenesis of cervical cancers. However, unlike cervical cancer, oral cancers are not widely accepted as a completely HPV associated malignancy. It is a well established fact that viral HPV DNA infection causes overexpression of E6 and E7 oncoproteins due to disruption of viral E2 gene. High risk HPV E7 in turn, is important for p16 up regulation by inactivation of pRB. Overexpression of p16 has been previously reported in HPV associated cancers which

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functions as a tumour suppressor binding to cyclin D1/CDK complex preventing the phosphorylation of pRB.

Several studies have consistently shown HPV infection and p16 immunoexpression to be independent favorable prognostic factors in head and neck cancers, especially among the oropharyngeal cancers (Begum et al., 2003; Weinberger et al., 2004; Reimers et al., 2007; Ang et al., 2010). Some studies have shown no prognostic relevance pertaining to loss of p16 expression (Geisler et al., 2002). HPV positive tumours have been previously characterized by high expression for p16. p16 immunoexpression has in fact been used as a surrogate marker for HPV infection in both oropharyngeal as well as non-oropharyngeal squamous cell carcinomas. However, it has also been indicated that p16 expression without HPV could be a distinct entity showing HPV presence does not always guarantee p16 overexpression as shown earlier and clinical behavior of p16 positive tumours lacking HPV is therefore not clear (Smeets et al., 2007; Fakhry et al., 2008; Stephen et al., 2013; Salazar et al., 2014).

Several studies have investigated the association of HPV infection in head and neck cancers with prevalence rates ranging from 0-100%. Such markedly different published reports of the HPV prevalence can be explained to be due to geographical differences between the studies, methodological differences in detecting HPV and sub site misclassification among others (Schwartz et al., 1998; Smith et al., 1998; Gillison et al., 2000). HPV is consistently detected in oropharyngeal cancers and tonsillar carcinoma which is about 5 times more than HPV prevalence in oral cavity and HPV 16 is the predominant subtype found (Herrero et al., 2003).

Although, HPV has been detected in varied amounts in persons with oral dysplasia, leukoplakia, and malignancy, its implication in oral tongue carcinogenesis remains ambiguous. In the current study, we sought to determine the role of HPV in pathogenesis, its concordance with p16 overexpression and assess the clinical behaviour of early staged oral tongue cancers. We have attempted to classify patients based on the HPV 16 and p16 status to evaluate the risk of poor outcome in our series of early staged tongue cancer.

Materials and Methods

Patient details: This study was approved by the University Research Council of the Tamil Nadu Dr. MGR Medical University to which the College of Oncological Sciences of the Cancer Institute (WIA) is affiliated. This is a retrospective study done in an exclusive cohort of early staged tongue cancer patients, (clinical stages I and II; T1-T2, clinically N0) at a tertiary cancer centre in Chennai, South India between 1995 and 2007. All the samples were histologically confirmed to be OTSCC before inclusion in this study. The study was carried out in paraffin blocks that was used for diagnostic purposes and for which a written informed consent was given by each of the patients as per the Institutional ethical guidelines. Every sample was verified for representation of the tumour in the biopsy with corresponding Haematoxylin and Eosin staining before including in the study. As per the ethical guidelines, patient anonymity was maintained in data management. DNA was isolated from representative formalin fixed paraffin embedded (FFPE) tongue cancer tissues (n=167) for the experimentation.

Clinical parameters and outcome assessment: All the patients underwent a routine evaluation which included a biopsy for histological confirmation of cancer, along with a comprehensive history and physical examination of upper aero digestive tract and neck imaging with ultrasound. Variables recorded and evaluated included age, sex, site, size of the tumour, pattern of the lesion, clinical stage, histological grade, tobacco with and without alcohol habits.

Treatment: The patients had undergone standard treatment consisting of either wide excision glossectomy or brachytherapy, with or without selective neck dissection (Levels I to IV). Patients unwilling/unfit for surgery were treated using External Beam Radiotherapy as per the decisions of multidisciplinary tumour board of the Institution. Pattern of failure and good outcome was recorded for each patient. Death due to the disease was included in the definition of the overall survival (OS), while time to disease recurrence in months was used to calculate disease free survival (DFS).

DNA Isolation: Formalin Fixed Paraffin Embedded (FFPE) samples were serially sectioned (10×5 μm) on a microtome and collected in a sterile tube for DNA isolation. Sectioning and sample preparation was performed to highest standards taking stringent measures to avoid contamination and cross-contamination between samples. The sections were deparaffinised and incubated at 56°C overnight with proteinase K solution, followed by heat-inactivation of proteinase K and DNA extraction, using the High Pure FFPET DNA isolation kit according to the manufacturer (Roche, Mannheim, Germany) recommendations. DNA quantity and quality was measured using Biophotometer plus (Eppendorf, Hamburg, Germany) and agarose gel electrophoresis and DNA isolates were frozen at -80°C until use.

Human Papilloma Virus detection by PCR: In all samples, the housekeeping gene β-globin gene was amplified first to confirm the adequacy of the extracted DNA. 11 samples did not amplify β-globin and were omitted from the analysis. The rest of the samples were tested for presence of HPV DNA by PCR using two sets of primers. The sequences for the SPF10 and GP5+/GP6+ primers are shown in (Table 1) (Snijders et al., 1990). The PCR reaction mixture of 25 μl included 1XPCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 40 pmoles of each primer, 0.5 μg/μl BSA, 1U Taq DNA polymerase and 100ng of genomic DNA. Cervical Cancer cell line SiHA, Caski and cervical cancer paraffin tissue genomic DNA that was known to harbour HPV previously were used as positive controls. Normal lymphocyte DNA was used a negative control. Amplification was performed with initial denaturing at 94°C for 5 minutes, followed by 42 cycles of 94°C for 1 minute, 40°C for 2 minutes and 72°C for 50 seconds and a final extension at 72°C for 4 minutes for GP5+/Gp6+ primers. For the SPF10 primers based HPV testing, amplification was done at initial denaturing at 94°C for 5 minutes, followed by 40 cycles of 94°C for

50 seconds, 50°C for 1 minute and 72°C for 30 seconds and a final extension at 72°C for 5 minutes. The PCR products were electrophoresed through 2 % agarose gel with ethidium bromide staining of PCR products visualized using ultra violet light and documented using gel doc (BioRad, U.S.A.).

Quantitative PCR to detect HPV 16 physical status: A TaqMan-based 5' exonuclease quantitative real-time PCR assay was used to determine the ratio of HPV16 E2 and E6 open reading frames (ORF) as described previously (Lee et al., 2010). Briefly, primers and probes specific for the E2 ORF and E6 ORF of HPV16 were used in the assay (Table 1). The 76-bp product of the E2 primers is part of the hinge region of the HPV16 E2 ORF, which is deleted during the viral integration process. 100ng of genomic DNA was used to set up a 20 µl PCR reaction with 1X TaqMan Mastermix (Applied Biosystems, Invitrogen, Carlsbad, CA, USA), 100 nM each of dual-labelled E2 and E6 probe and 100 nM of E2 and 75nM of E6 primers (Shrimpex Biotech Services Ltd., Chennai, India). Threshold cycle numbers (Ct) were determined with 7500 real-time PCR System (Applied Biosystems, Invitrogen, Carlsbad, CA, USA) and the copy numbers were estimated by absolute quantification by standard curve method.

A six-point dilution series of CaSki cell line genomic DNA, which is known to have 600 copies of HPV16/ genome equivalents, was used to obtain standard curves for E2 and E6. The physical status of HPV16 viral genome was determined by calculating the ratio of E2 to E6 copy numbers. E2/E6 value of 0 represented presence of only integrated virus, E2/E6=1 indicated presence of episomal HPV virus and a E2/E6 value between 0 and 1 signified the concurrence of both episomal and integrated viral forms. Genomic DNA from SiHa cell line which contains a pure, integrated form of the HPV16 gene was used as negative control for E2 amplification and positive control for E6 amplification. HeLa cell line genomic DNA which contains HPV18 was used as negative control for HPV 16 E2 and E6.

Table 1. Primer and Probe Sequences Used for HPV Studies

Primer Name	Primer Sequences
β- Globin Sense	5'ACACAACTGTGTTCACTAGC '3
β- Globin Antisense	5'CAACTTCATCCACGTTCACC '3
GP5+ Sense	5'TTTGTTACTGTGGTAGATACTAC '3
GP6+ Antisense	5'GAAAAATAAACTGTAAATCATATTC '3
SPF 10 Forward	5'GCiCAGGGiCACAATAATGG '3 5'GCiCAGGGiCATAACAATGG '3 5'GCiCAGGGiCATAATAATGG '3 5'GCiCAAGGiCATAATAATGG '3
SPF 10 Reverse	5'GTiGTATCiACAACAGTAACAAA '3 5'GTiGTATCiACTACAGTAACAAA '3
HPV16 E2 Probe	5'(JOE) - CACCCCGCCGCGACCCATA- (BHQ1) 3'
HPV16 E2 Forward	5'AACGAAGTATCCTCTCCTGAAATTATTAG 3'
HPV16 E2 Reverse	5'CCAAGGCGACGGCTTTG 3'
HPV16 E6 Probe	5'(6-FAM)- AGGAGCGACCCAGAAAGT TACCACAGTT- (BHQ1) 3'
HPV16 E6 Forward	5'GAGAACTGCAATGTTTCAGGACC 3'
HPV16 E6 Reverse	5'TGTATAGTTGTTTGCAGCTCTGTGC 3'

p16 Immunohistochemistry(IHC): The IHC detection of p16 expression was performed on five-micron sections of FFPE tissues. The sections were deparaffinised in xylene and rehydrated in absolute ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in Phosphate- buffered Saline (PBS) for 30 minutes and subjected to antigen retrieval in Tris-EDTA buffer (pH-8) by autoclaving at 121°C for 10 minutes. Sections were pre-incubated in 2% Bovine Serum Albumin (BSA) for 30 minutes and then incubated with mouse monoclonal antibody against p16 (clone - JC8) (sc-56330) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) in 1:150 dilution, overnight at 4°C. p16 expression was observed using the SuperSensitive™ Polymer-HRP IHC Detection System (BioGenex Laboratories, San Ramon, CA). Sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. Positive controls included sections of cervical cancer previously known to overexpress p16 tested by IHC. Primary antibody was replaced with 2% BSA in negative control. p16 immuno-expression was scored as described before (Zhao et al., 2012). Briefly, each sample was given a cytoplasmic as well as nuclear intensity score on a scale of 0-3. The percentage of tumour cells with positive nuclei was determined by scoring 10 microscopic fields of 100 tumour cells each. A semi-quantitative approach was opted to give the p16 scores based on percentage of tumour cells expressing p16. Scale 1-faint or low cytoplasmic and nuclear staining (LS) in less than 20% of tumour cells, Scale 2-High cytoplasmic and low nuclear staining (HC) in less than 50% of tumour cells and Scale -3-High nuclear and high cytoplasmic staining in greater than 50% of tumour cells. We considered Scale 3 tumors showing intense nuclear and cytoplasmic staining as positive for p16 expression (Jayasurya et al., 2005).

Statistical Analysis: All statistical analyses were carried out in SPSS version 16.0. Distribution of categorical variables was compared by Pearson's Chi-squared test or Fischer's exact test according to the counts of expected frequencies. Estimated survival curves were obtained by Kaplan Meier method and the results were compared using log rank test. Logistic regression analysis was done to determine the significant variables to predict outcome, survival and recurrence. To analyse the prognostic factors for the risk of recurrence and death, the patient groups and clinico-pathological characteristics were evaluated for association with time to recurrence and death using the Cox proportional hazards regression model. A hazard ratio with 95% confidence intervals (CIs) from Cox Model was obtained by Univariate analysis. For multivariate analysis, the factors for which p value was below 0.1 in univariate analysis were selected and model was developed based on forward likelihood ratio method to derive significant prognostic variables. Statistical significance was given to the p values <0.05.

Results

Our study included 167 patients with histologically confirmed Oral tongue cancers and all the patients belonged to early stage (T1-T2, clinically N0). We report

findings for 156 patients who had acceptable quality of DNA for experimentation based on status of housekeeping gene β globin. Median follow up period was 74 months, median recurrence free survival duration was 21.5 months.

High HPV DNA prevalence in early staged oral tongue squamous cell carcinoma: Of 167 oral tongue cancer samples taken for the studies, 11 samples (6.5%) did not amplify for β globin PCR despite repeated efforts, showing a poor quality of DNA and hence were excluded from the study. Of the remaining 156 samples taken for HPV DNA evaluation, we found HPV DNA prevalence in 81/156 (52%) by GP5+/GP6+ and SPF 10 consensus PCR. (Figure 1a) All the samples were further evaluated for HPV16 DNA prevalence and their physical status using the qPCR. HPV 16 DNA was found in 69/81 (85.18) of samples (Figure 1 a) Patient demographic data represented based on HPV status is shown in Table 2.

Table 2. Clinico-Pathological Variables Classified By HPV DNA Prevalence

Variables	Total (n=156)	HPV DNA Negative (n=75)	HPV DNA Positive (n=81)
Age			
<45 Years	43	21 (48.8)	22 (51.2)
46-65 Years	89	39 (43.8)	50 (56.2)
>65 Years	24	15 (62.5)	9 (37.5)
Gender			
Male	108	52 (48.2)	56 (51.8)
Female	48	23 (47.9)	25 (52.1)
Site			
Lateral Border	138	68 (49.3)	70 (50.7)
Tip	3	2 (66.7)	1 (33.3)
Dorsum	5	2 (40)	3 (60)
Ventral Aspect	10	3 (30)	7 (70)
Clinical Stage			
Stage I	61	30 (49.2)	31 (50.8)
Stage II	95	45 (47.4)	50 (52.6)
Size			
0-2 cm	62	31 (50)	31 (50)
2.1-3 cm	74	34 (45.9)	40 (54.1)
> 3 cm	20	10 (50)	10 (50)
Pattern			
Exophytic	40	18 (45)	22 (55)
Infilrating	93	45 (48.4)	48 (51.6)
Ulcerated	23	12 (52.2)	11 (47.8)
Pathological Grade			
Well Differentiated	117	63 (53.8)	54 (46.2)
Moderately Differentiated	21	4 (19)	17 (81)
Poorly Differentiated N	7	2 (28.6)	5 (71.4)
			p=0.008
Tobacco Habits			
Chewing	40	18 (45)	22 (55)
Smoking	29	9 (31)	20 (69)
Chewing + Smoking	18	9 (50)	9 (50)
Chewing + Smoking + Alcohol	87	36 (41.4)	51 (58.6)
Non User	69	39 (56.5)	30 (43.5)
HPV16 DNA			
HPV16 Negative	87	75 (86.2)	12 (13.8)
HPV16 Positive	69	0	69 (100)
Physical Status of HPV 16			
Episomal	23	0	23 (100)
Integrated	21	0	21 (100)
Mixed	25	0	25 (100)
p16 Expression			
Negative	132	63 (47.7)	69 (52.3)
Positive	24	12 (50)	12 (50)
Management			
Brachytherapy	104	48 (46.2)	56 (53.8)
Radiotherapy	42	21 (50)	21 (50)
Surgery	10	6 (60)	4 (40)
Upfront Neck Node Management			
Observation	77	36 (46.8)	41 (53.2)
Neck Dissection	37	18 (48.6)	19 (51.4)
Radiation to neck nodes	42	21 (50)	21 (50)

HPV status correlates with histological grade in early staged OTSCC

HPV status was found to be associated with grade of the tumour (p=0.008). Moderately and poorly differentiated tumours had increased prevalence of HPV compared to well differentiated tongue tumours. Logistic regression analysis showed tumour grade as a significant covariate in our series predicting HPV DNA prevalence (OR= 3.050; 95% CI -1.377-6.756). (Data not shown)

Presence of HPV DNA and its physical status is not associated with treatment outcome in OTSCC

The evaluation of the physical HPV 16 DNA status

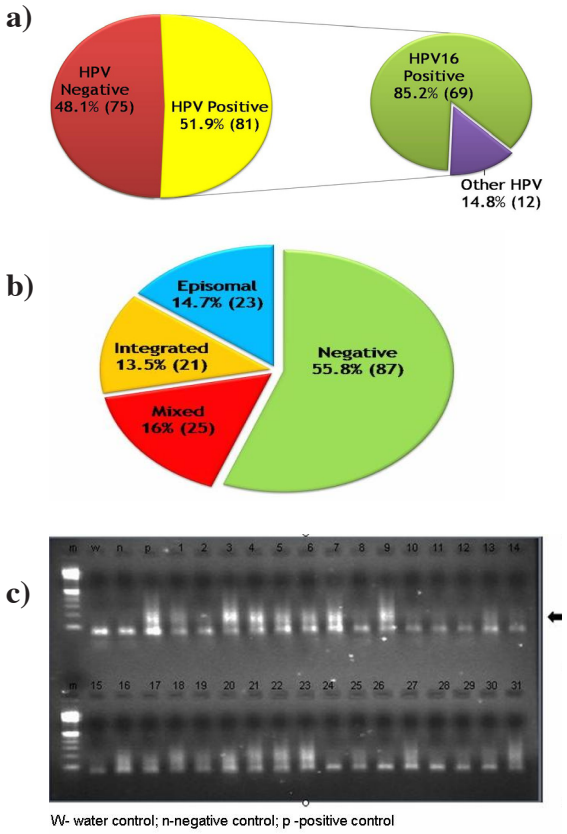

Figure 1 HPV ASsessment. a) HPV DNA Prevalence; b)Physical Status of HPV 16 DNA; c) HPV PCR Products Using GP5+ GP6+ Primers from Archived Paraffin Embedded Samples

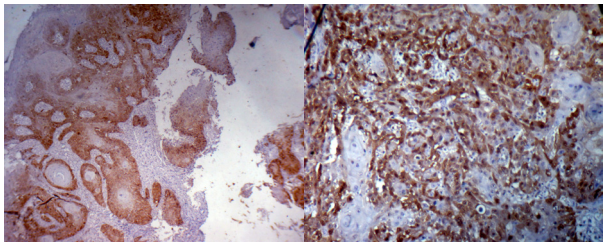

Figure 2. Well Differentiated Squamous Cell Carcinoma of Oral Tongue showing Dense p16 Staining in >50% of Cellular Nucleus and Cytoplasm. A) (IHC 10X)**; B)** (IHC 40X)

showed 23/69 (33.3%) in episomal form, 21/69 (30.4%) in the integrated form and 25/69 (36.2%) in the mixed form containing both episomal and integrated forms (Figure1 b). HPV DNA prevalence and HPV16 infection were not found to be significantly associated with treatment outcome in our series of early staged oral tongue cancer patients.

Presence of HPV DNA is not concordant with corresponding p16 expression in OTSCC

p16 expression was negative in a majority of the cases (84.6%; 132/156). Intense and diffuse nuclear and cytoplasmic expression for p16 was identified in 24/156 (15.3%) of oral tongue tumours (Figure 2a and 2b) (Table 2). The concordance between intense and diffuse p16 expression in greater than 50% of the tumour nuclei along with HPV prevalence was observed in only 10/81 (12.3%) tumour samples with a poor kappa value < 0.2.

Comparing HPV 16 and p16 concordance, we had 14/87 (16%) tumours with intense p16 overexpression without HPV 16 infection (p16+; HPV16-), 59/69 (85.5%) of tumours with HPV 16 DNA not expressing p16 (HPV16+; p16 -) and we had 73/87 (83.9%) of tumours without HPV 16 DNA also negative for p16 expression (HPV16-; p16 -).

P16 expression is not a suitable marker indicating presence of HPV infection in OTSCC

We evaluated, if expression of p16 (both focal as well as intense and diffuse expression) could predict the HPV 16 DNA prevalence in tongue cancers, and found the sensitivity of p16 expression to indicate HPV 16 infection in our series of cases to be 53% (95 % CI ; 0.41-0.65) and specificity to be 50 % (95% CI ; 0.39-0.61). The positive predictive value (PPV) and Negative predictive value (NPV) was 48% (95% CI 0.37-0.60) and 55% (0.43-0.66) respectively. Due to less sensitivity and specificity for high risk HPV presence, p16 expression may not be suitable surrogate marker for HPV 16 infection in oral tongue cancers.

P16 expression indicates poorer outcome and survival in OTSCC

To evaluate significant co-variates to predict treatment outcome, Overall Survival (OS) and disease free survival (DFS) for our series of samples (n=156), we did binary logistic regression analysis (Table 3). The analysed characteristics included age, sex, clinical stage, grade, pattern, treatment modality, upfront management of neck nodes, HPV 16 infection, p16 expression, tobacco with or without alcohol related habits.

Significant co-variates for poorer outcome were p16 overexpression (OR=4.711; p=0.021). exophytic pattern of lesion (OR=0.526; p=0.028), upfront neck node management (OR=1.905; p=0.006). Significant covariates for risk of death were increased age (OR=1.948; p=0.026), female sex (OR=2.327; p=0.031), Increased stage (OR=2.907; p=0.007), tobacco habits (OR= 1.390; p=0.031) and p16 overexpression (OR=3.083; p=0.045). Significant covariate for risk of recurrence was p16 overexpression (OR=4.722; p=0.008). p16 therefore was found to be a significant predictor of poorer outcome with increased risk of death and recurrence.

Cumulative hazard of OTSCC related mortality and recurrence was increased among patients with tumours showing intense and diffuse nuclear and cytoplasmic expression of p16, which was statistically significant (log rank test p=0.005 and p=0.000) (Figure 3a and b)

Prognostic variables for OTSCC by Cox proportional Hazard Model

Significant prognostic variables for OS and DFS were derived using Cox proportional hazards model. Adjusted Cox models for prognostic variables for OS and DFS are shown in Table 4. Absence of HPV 16 DNA indicated marginal hazard of death in the Multivariate Cox model (HR=0.613; p=0.049). HPV 16 DNA however was not significant predictor for DFS and disease outcome. We found p16 over expression to be a significant poor prognostic indicator for increased hazard of death (HR=2.395; p=0.005) and increased hazard of disease recurrence (HR=2.581; p=0.002). Interestingly absence of HPV 16 and presence of p16 overexpression were indicating poorer prognosis.

Risk Stratification shows p16 overexpression as an indicator of high risk of mortality and recurrence irrespective of HPV and tobacco and/or alcohol related habits

Table 4. Prognostic Factors by Multivariate Cox Hazard Model

Variables	OS		DFS	
	HR	p value	HR	p value
Age	1.586 (1.089-2.310)	0.016		
Sex	1.632 (1.007-2.644)	0.047		
Stage	2.402 (1.078-5.352)	0.032		
P16	2.395 (1.303-4.403)	0.005	2.581 (1.436-4.637)	0.002
HPV16 DNA absence	0.613 (0.376-0.997)	0.049		

Table 3. Identification of Risk Factors by Binary Logistic Regression

Variables	Treatment Failure Odds Ratio	Risk of Death Odds Ratio	Risk of Recurrence Odds Ratio
Age	-	1.948 (1.085-3.495) P=0.026	-
Sex	-	2.327 (1.027-5.021) P=0.031	-
Stage	-	2.907 (1.335-6.327) P=0.007	-
Tobacco Habits	-	1.390 (1.031-1.874) P=0.031	-
P16 overexpression	4.711 (1.261-17.599) P=0.021	3.083 (1.024-9.285) P=0.045	4.722 (1.510-15.083) P=0.008
Pattern	0.526 (0.297-0.933) P=0.028	-	-
Upfront neck node management	1.905 (1.207-3.004) P=0.006	-	-

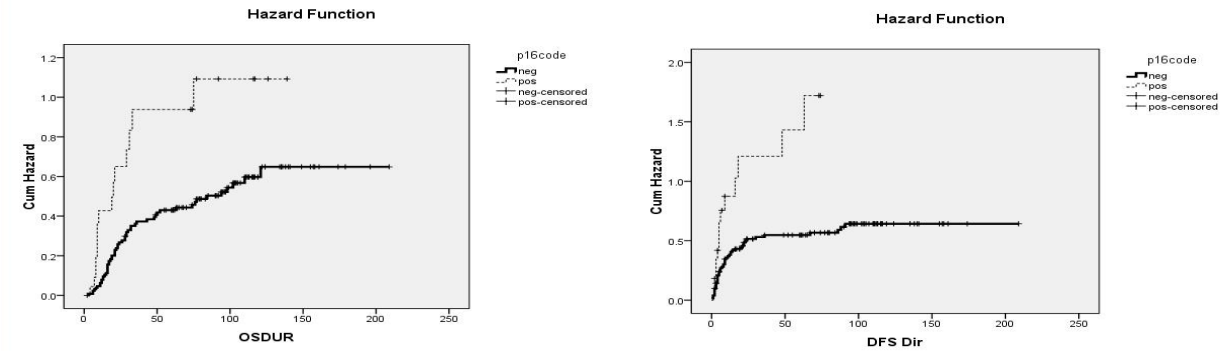

Figure 3. Cumulative Hazard for Cancer. A) specific mortality based on intense and diffuse p16 expressioncompared by log rank test (p value =0.006); **B)** recurrence based on intense and diffuse p16 expression comparedby log rank test (p value= 0.000)

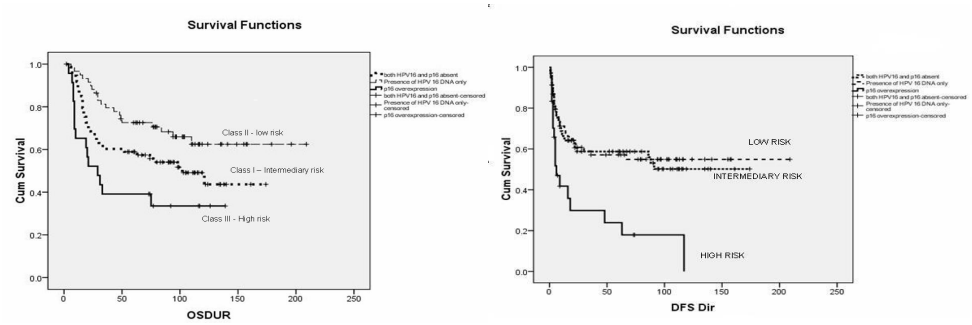

Figure 4. Kaplan Meier Survival Plots Comparing. A) Overall Survival by Log Rank Test (p value =0.004); **B)** DFS by Log Rank Test (p value =0.001)

Kaplan Meier survival plots comparing the overall survival and disease free survival fractions of the above mentioned groups are shown in Figure 4 a and b. There was a significant poorer OS (Log rank p value=0.004) and poorer DFS (Log rank p value=0.001) among the patients whose tongue tumours overexpressed p16 showing the worst outcome, classifying them as the high risk group in the current series. Patients whose tumours showed prevalence of HPV 16 DNA alone without p16 had the best OS thus classified as low risk group. The percent survival fraction was 66.1% for patients whose tumours had HPV 16 DNA alone without p16 expression classified as low risk. The intermediary risk group of patients with no HPV 16 DNA and no p16 expression had 50.7% survival fraction. Patients with p16 overexpression had a survival fraction of 35.7% classified as high risk. We had 14 patients (58.3%) with p16 overexpression not harboring HPV DNA indicating a probable HPV independent mechanism for p16 expression in these patients.

The OS and DFS were very poor for this cohort of patients within the p16 overexpressing group with surviving fractions of 35.7% and DFS of 18.2% respectively. Evaluating the tobacco and alcohol related habits in this group, we found that we had (54.1%; 13/24) with habits and (45.8%; 11/24) without habits indicating importance of p16 overexpression irrespective of presence or absence of habits.

Discussion

Our study done on exclusive sub site oral tongue shows a different perspective pertaining to the role of p16 and HPV DNA prevalence and has helped classify our series of patients based on risk of death and recurrence. Earlier reports from India in largest series of OSCC (n=348) had only 27.2% tongue cancers (95/348) (Jayasuriya et al., 2005).

HPV prevalence: In the present study, we report that the prevalence of HPV DNA studied using PCR in OTSCC is high (51.2%). Earlier reports from India on HPV prevalence studied by PCR in exclusive oral tongue sub site (n=60) was 50% (Elango et al., 2011) and our reports are similar to this study. PCR and real time PCR based detection of HPV is expected to be high owing to the sensitive technology used. Another study using PCR based method has shown a prevalence of 32% (Debolina Pal et al., 2007). Biological relevance of HPV would be more appropriately derived using mRNA quantitation from the preferably fresh tissues.

The prevalence of HPV reported from 13 studies from oral cavity in the Asian subcontinent have reported a prevalence of about 33.3%, of which the rates of HPV 16 prevalence was reported to be 22.3% which is much higher compared to other parts of the world. Despite the higher prevalence, the probably role of HPV is reported 5 times lower than oropharyngeal cancers (Combes and Franceschi, 2014). The prevalence of HPV in normal oral mucosa, oral potentially malignant disorders and oral malignancies varied from 0 to 70%, 0 to 85%, and 0 to 100%, respectively (Ha and Califano, 2004). A high prevalence of HPV in oral cancers from India has been reported previously, suggesting that viral agents could cause additional mutations in the carcinogenic process, together with dietary habits and probably, in the presence of a given genetic predisposition (Balaram et al., 1995). A meta-analysis by Termine et al. showed that overall

prevalence of HPV DNA in oral SCC was 38.1% (Termine et al., 2008). With regards to the detection method, PCR-based studies reported a higher prevalence rate than ISH-based rates (39.9% versus 32.9%). In particular, this rate was higher than that reported before (Kreimer et al., 2005). A recent study has shown that topography plays a role in HPV prevalence in oral lesions. Even though the same lesions were found in different regions of the oral cavity, the HPV positivity was higher in specific topographical regions irrespective of diagnosis. A large discrepancy of observed HPV prevalence in the data available can be due to analyzing samples of a particular diagnosis taken from different regions of oral cavity, thus reaching different conclusions (Mravak-Stipetic et al., 2013). The current controversy regarding the role of HPV in oral carcinogenesis is therefore justified (Boy et al., 2006). A study on HPV prevalence in oral cavity cancer (including lip, buccal mucosa, gingivobuccal complex along with anterior 2/3rd tongue) from another Indian study was reported to be around 34% (Koppikar et al., 2005). Reports from non-Indian population from in exclusive cohort of OTSCC has reported HPV prevalence as 11.3%, of which 7.5% is HPV 16 (Tsimplaki et al., 2014). In the current study we report HPV 16 as the most common HPV infection as reported in many earlier studies (Kreimer et al., 2005).

HPV 16 and p16 concordance: In our study cohort, a majority of OTSCCs did not express p16 (84.6%) as was also the case in previously reported studies (Lim et al., 2014). Our study showed a very poor concordance between HPV 16 DNA and p16 IHC with a kappa less than 0.2. These findings are similar to the reports of Liang et al. who further showed that the concordance slightly improved if HPV serology was compared to p16 IHC (Liang et al., 2012). We therefore report that p16 overexpression may not be a suitable surrogate marker for HPV infection in tongue cancers. Poor positive predictive value for detection of HPV infection by p16 expression has been reported earlier (Lewis et al., 2010; Lingen et al., 2013) similar to the current study. These results are also supported in large meta-analysis study reported in non-oropharyngeal cancers (Combes and Franceschi, 2014).

Promoter methylation of p16 should be evaluated in this cohort of patients showing negative immunoexpression for p16. Quantitative PCR, being the most sensitive method available, can help in identifying the physical status as well. The physical status of the high risk HPV 16 DNA however, was not found to be associated with disease outcome in our series of cases.

Risk stratification: Our studies for the first time shows patients with tumours showing overexpression of p16 (high risk) having worst outcome, irrespective of presence or absence of HPV 16 DNA compared to patients with tumours with HPV 16 DNA positive alone without p16 expression (low risk) having the best outcome. We hypothesize that, among patients with HPV16 DNA alone and absence of p16 expression (low risk group) their HPV 16 prevalence does not probably indicate transcriptionally active state of HPV. We also show that p16 expression and HPV infection are not concordant in oral tongue cancers. In oropharyngeal cancers, the concordance of p16 and HPV is reported higher and p16 positive and HPV positive tumors have significantly better prognosis. p16 IHC as a surrogate marker for HPV infection has been depicted in several studies (Wittekindt et al., 2005; Lewis et al., 2010; Oguejiofor et al., 2013) and is considered as a molecular hall mark of HPV positive HNSCC (Klussmann et al., 2003; Li et al., 2004).

We hypthesise that unlike oropharyngeal cancers, HPV probably does not a have a meaningful role to play in oral tongue cancers since transcriptional activity in the form of p16 expression was found to be absent in majority of cases.

HPV prevalence studies in tongue cancers could possibly be attempted by other methodologies like E6 and E7 serology to understand its biological relevance. Recent study using massive parallel sequencing of tongue cancers has also shown absence of significant viral transcripts (Bragelmann et al., 2013).

We have shown that p16 overexpression indicates poorer outcome despite the presence or absence of HPV infection. Previous studies have shown p16 expression independent of HPV infection to be associated with epithelial to mesenchymal transitions mediated by MAPK pathway (Steinestel et al., 2013). Our report is also similar to recent studies showing the possibility of p16 overexpression without HPV presence in tumours concluding that it need not always be interpreted as a defacto HPV marker (Alexander et al., 2014; Kim et al., 2014). Our results are in contrast to findings reported by Konig et al., suggesting that p16 immunostaining is to be applied as a pre-screening method for HPV subtyping and that p16 IHC correlates with high risk HPV status (Konig et al., 2007). However a majority of the cases reported in this study were primaries from larynx and only 19 samples were from the oral cavity. However this may not be applicable to tongue cancers where we report poor positive predictive value for p16 in detection of HPV in oral tongue cancers, which is similar to reports of Lingen et al. (Lingen et al., 2013).

In conclusion, our study has brought out several key facets which can potentially redefine our understanding of tongue cancer tumorigenesis. Interestingly, the prognostic significance of p16 overexpression is different from that reported in oropharyngeal cancers. This study done in an exclusive non-oropharyngeal subsite, tongue has shown that HPV may not have a significant role to play in oral tongue cancers and p16 may not be considered as a suitable HPV surrogate marker. We report the role of p16 as a single important prognostic variable defining high risk group and depicting poorer prognosis. The study highlights the need for its routine assessment and indicates a probable HPV independent mechanism leading to its activation like EMT pathways or increased MAP kinase pathway that needs to be probed.

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